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The Role of Kindlin-1 in Pulmonary Breast Cancer Metastasis

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Abstract

Kindlin-1 is a focal adhesion (FA) protein and the founding member of the Kindlin family of proteins (Kindlins 1-3), which all have an important role in integrin signalling. Loss of functional Kindlin-1 has been associated with a congenital disease called Kindler syndrome that is characterised by a number of skin abnormalities including atrophy and blistering. Increased or decreased expression of the Kindlin family of proteins has been linked to a number of tumour types and Kindlin-1 is specifically associated with pulmonary metastasis in basal-like breast cancer. The mechanisms by which Kindlin-1 mediates metastasis to the lungs in breast cancer remain relatively unknown and this study aimed to investigate the role of Kindlin-1 in breast cancer. In line with previous reports, we have shown that Kindlin-1 regulates primary breast tumour formation. Our data suggest that the interaction of Kindlin-1 with integrins is specifically important for tumour initiation.

The Kindlins have also been shown to have roles independent of integrin and have been found outside of focal adhesions; Kindlin-2 has been shown to have a nuclear localisation signal (NLS) and initial studies have identified a nuclear role for Kindlin-2 in the promotion of breast cancer, however, nuclear roles of the Kindlins remain mostly unexplored. We explored a potential nuclear role for Kindlin-1 and revealed a novel cellular location for Kindlin-1 in cancer cells. We show that Kindlin-1 is present in the nucleus of breast cancer and squamous cell carcinoma cells and that Kindlin-1 has an NLS that can be altered to prevent protein localisation to the nucleus. Furthermore, we show that presence of Kindlin-1 in the nucleus is important for primary tumour growth and mice inoculated with cells that express mutant Kindlin-1 that cannot localise to the nucleus, have significantly lower tumour growth compared to mice inoculated with cells expressing wild-type Kindlin-1. We also provide evidence that the immune system impacts on the Kindlins ability to regulate tumour growth. Specifically, Kindlin-1 was shown to inhibit IL-6 production, which promotes differentiation of naïve CD4+ T cells into Tregs and results in tumour development due to suppression of the anti-tumour response of the immune system. Our findings

are particularly interesting as the FA protein FAK has previously been reported to have an immune-modulatory role in the nucleus of squamous cell carcinoma cells through regulation of the transcription of inflammatory cytokines and chemokines. Our data suggest that Kindlin-1 may also have similar important roles in the nucleus that warrants further investigation. As Kindlin-1 does not have catalytic activity, further studies are warranted to investigate the nuclear roles of Kindlin-1 and the interaction of Kindlin-1 with the immune system, as understanding these roles may offer new avenues for therapeutic intervention.

Very few binding partners have been identified for the Kindlin family and known binding partners include phosphoinositides and some focal adhesions proteins such as integrin-linked kinase (ILK) and migfilin. We carried out proteomic analyses and identified a number of novel binding partners of Kindlin-1 in different breast cancer cell models. Our findings suggest that Kindlin-1 is an adaptor protein in the cell that regulates a number of key signalling hubs, which control several cellular processes including metabolism, cell proliferation, cell migration, apoptosis and signal transduction.

Lay Reader Summary

Breast cancer is the most common type of cancer diagnosed in women worldwide. Most breast cancer deaths are a result of the spread of cancer from the breast to other parts of the body. Increased levels of Kindlin-1, a protein involved in cell contacts with their environment, has been associated with breast cancer and its spread to the lungs. How Kindlin-1 promotes breast cancer is mostly unknown. The aim of our study was to explore the role of Kindlin-1 in breast cancer.

We wanted to understand when Kindlin-1 is important in breast cancer growth and spread. We found when the production of Kindlin-1 was stopped, tumour growth was reduced. Our research has shown that when tumours are first growing, Kindlin-1 helped tumours to grow by interacting with other proteins involved in cell to cell contacts called integrins. However, once tumours were established Kindlin-1 helped tumours to grow without using integrins. We also found evidence that Kindlin-1 may be involved in controlling the immune system. The immune system is important for slowing or preventing tumour growth and so this interaction is important.

Kindlin-1 has not been shown to interact with many proteins other than integrins. We aimed to identify new interactions that might be important in the progression and spread of breast cancer. We identified a number of proteins that interact with Kindlin-1 in breast cancer cells. Our findings suggest Kindlin-1 interacts with a number of different proteins, linking Kindlin-1 to cellular processes including cell movement, death and proliferation of cells.

Another protein that has similar roles to Kindlin-1, Kindlin-2, has been found in the nucleus in breast cancer. We identified that Kindlin-1 contains a signal that tags it for transportation into the nucleus and that Kindlin-1 was able to move into the nucleus of cancer cells. We demonstrated that cancer growth reduces when Kindlin-1 was prevented from moving to the nucleus. We, therefore, suggest that Kindlin-1 might control which genes are used by tumour cells in order to grow. Further studies should investigate the role of Kindlin-1 in the nucleus and its interaction with the immune

system. Understanding these roles may identify specific targets for drugs to be more effective in the treatment of breast cancer.

Declaration

I declare that this thesis has been composed by myself and details my own research unless stated otherwise in the text. No part of this thesis has been submitted elsewhere for any other degree or personal qualification except as specified.

Georgia Louise Dodd

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List of Abbreviations

Abbreviation	Meaning
ACLY	ATP-citrate lyase
ALDH18A1	Aldehyde dehydrogenase 18 family member A1
APC	Adenomatous polyposis coli
CDKN1A	Cyclin-dependent kinase inhibitor 1A
CDK	Cyclin-dependent kinase
CHO	Chinese Hamster Ovary
CSCs	Cancer stem cells
CSL	CBF1 suppressor of hairless, Lag-1
CTCs	Circulating tumour cells
Dox	Doxycycline
DMSO	Dimethyl sulfoxide
DNMT3A	DNA methyltransferase 3 alpha
Dync1h1	Dynein Cytoplasmic 1 Heavy Chain 1
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Estrogen
FGF	Fibroblast growth factor
FA	Focal adhesion
FAK	Focal Adhesion Kinase
FERM	Four-point-one, ezrin, radixin, moesin
GSI	γ-secretase inhibitor
HADHA	Hydroxyacyl-CoA dehydrogenase trifunctional multi-enzyme complex
H&E	Haematoxylin and Eosin
HER2	Human epidermal growth factor receptor 2
HSPs	Heat shock proteins
HSPH1	Heat shock protein family H member 1

IF	Immunofluorescence
IHC	Immunohistochemistry
ILK	Integrin-Linked Kinase
JAK	Janus-activated kinase
KS	Kindler Syndrome
LAP	Latency-associated peptide
LGR5	Leucine-rich repeat-containing G-protein-coupled receptor 5
LPA	Lysophosphatidic acid
LTBP	Latent TGF-β binding protein
MaSCs	Mammary stem cells
MCM	Mini-chromosome maintenance
MMTV	Mouse mammary tumour virus
MMTV-PyMT	Mouse mammary tumour virus-polyoma middle tumour-antigen
MSCs	Mesenchymal stem cells
MSI1	Musashi homolog 1
MT	Microtubules
MTHFD1	Methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1
NF-κB	Nuclear factor-κB
NLS	Nuclear localisation signal
NOD SCID	Non-obese diabetic severe combined immune deficient
NSCs	Normal stem cells
NSUN2	NOP2/Sun RNA methyltransferase 2
PARP	poly(ADP-ribose) polymerase
PH	Pleckstrin homology
PI3K/PTEN	Phosphatidylinositol 3-kinase/phosphatase and tensin homolog
Plk-1	Polio-like kinase
PMSC	Proteasome 26s sunbunit ATPase
PR	Progesterone
PROCR	Protein C receptor
PTB	Phosphotyrosine-binding

RFP	Red fluorescent protein
RT-qPCR	Real-time quantitative reverse transcription PCR
SARA	Smad anchor for receptor activation
SCC	Squamous cell carcinoma
SEM	Standard error
SHH	Sonic hedgehog
STAT	Signal transducer and activator of transcription
TβRI	TGF-β receptor I
TCF4	T-cell factor 4
TCP	T-complex 1
Tet	Tetracycline
TGF-β	Transforming growth factor β
TH17	T helper 7
TNBC	Triple negative subtype of breast cancer
TNFα	Tumour Necrosis Factor alpha
Tregs	Regulatory T cells
tRFP	Turbo red fluorescent protein
Tubb4b	Tubulin Beta 4B Class IVb
Tubb6	Tubulin Beta Class V
TUFM	Tu translation elongation factor, mitochondrial
VCP	Valosin-containing protein
VEGF	Vascular endothelial growth factor

Chapter 1 Introduction

1.1 Breast Cancer

Breast cancer is the most common type of cancer diagnosed in women worldwide and is responsible for 7% of all cancer related deaths in the UK (1). The disease can be ductal or lobular in origin and 80% of invasive ductal carcinoma accounts for diagnosed breast cancer cases (2). Invasive breast cancer has been classified into both histological and intrinsic molecular subtypes, owed to the complexity and heterogeneity of the disease (3). Broadly, breast cancer has been classified into four molecular subtypes and these include: luminal A, luminal B, HER2-enriched, basal-like (Table 1), which have been identified by techniques such as immunohistochemistry (IHC) and gene expression profiling (4–6). Treatment for patients with cancer has been stratified using a number of factors including tumour morphology and grade classification, tumour size, presence of lymph node metastases, and expression of estrogen (ER), progesterone (PR) and human epidermal growth factor receptor 2 (HER2) receptors.

Table 1. Intrinsic molecular subtypes of invasive breast cancer

Intrinsic Subtype	Protein Expression Profile
Luminal A	ER+ and/or PR+, HER2-, Ki67-
Luminal B	ER+ and/or PR+, HER2±, Ki67+
HER2 over-expression	ER-, PR-, HER2+
Basal-like or Triple Negative	ER-, PR-, HER2-, basal marker +

ER- oestrogen receptor; HER2- human epidermal growth factor receptor 2; PR- progesterone receptor

The ER and PR hormone receptors have been known to drive progression of cancer for some time and up to 80% of breast cancers are identified as ER-positive (7) whereas, 55-65% are PR-positive (8). The luminal A and B subtypes display hormone receptor positivity and as a result, have better outcomes than HER2 and basal/triple

negative subtypes because hormone therapy can be used to treat these tumour types (1). As well as receptor status, the marker of cell proliferation, Ki67 is used to categorise luminal A and B tumours and high levels of Ki67 are associated with worse outcome (9,10). Luminal A has been associated with 30-40% of invasive breast cancers and luminal B HER2- with 20-30% of cases (11).

The HER2 subtype, which accounts for 15-20% of breast cancers (2,12), is characterised by high levels of a transmembrane tyrosine kinase receptor that regulates cell growth, proliferation and survival via signalling pathways including mTOR and RAS/RAF/MEK/ERK (13). Tumours of this molecular subtype respond to anti-HER2 therapy and agents that target HER2 and these include: receptor tyrosine kinase inhibitors and humanised monoclonal antibodies that bind to the extracellular domain of HER2 receptors.

The basal-like or triple negative subtype of breast cancer (TNBC) is characterised by lack of expression of hormone receptors or HER2 and high expression of basal markers (e.g. keratins 5, 6, 14, 17) (4,14). These account for 15-20% of breast cancer cases and have the worst survival rates compared with other subtypes (11). A study showed that the 5-year survival rate for patients in the TNBC subgroup was 77% whilst the 5-year survival was 93% for non-TNBC patients (15). While treatments exist for receptor positive subtypes of breast cancer, there are no treatments to specifically target TNBC. Treatment is limited to surgery, radiation and chemotherapy, however, some breast cancers with the BRCA 1 or 2 mutation have a basal/TNBC pathology and respond better to cisplatin-based chemotherapy and breast cancer poly(ADP-ribose) polymerase (PARP) inhibitors when compared to BRCA wild-type tumours (16,17).

Although there have been advances in diagnosis and treatment as a result of our improved understanding of the molecular heterogeneity of breast cancer, mortality

and other complications related with breast cancer are a result of the development of incurable metastatic disease (18). In fact, over 90% of cancer-related death is a result of metastatic disease (19). Breast cancer typically metastasises to the brain, bones, liver or lungs (20). Approximately 30% of breast cancer patients develop recurrences in distant organs despite successful treatment of the primary tumour (21). Recurrences often occur years after the primary tumour is diagnosed, which suggests that patients carry dormant tumour cells that later progress to become metastases (Fig. 1A) (21). There have been several hypotheses to describe how tumour cells survive and remain in a dormant state then later become reactivated and form macrometastases. The mechanisms of dormancy include cellular dormancy (Fig. 1B), insufficient angiogenic potential (Fig. 1C), active immune surveillance (Fig. 1D), lack of tumour cell adhesion to the extracellular matrix (ECM) and interaction with stromal cells, which secrete anti-proliferative factors (22,23).

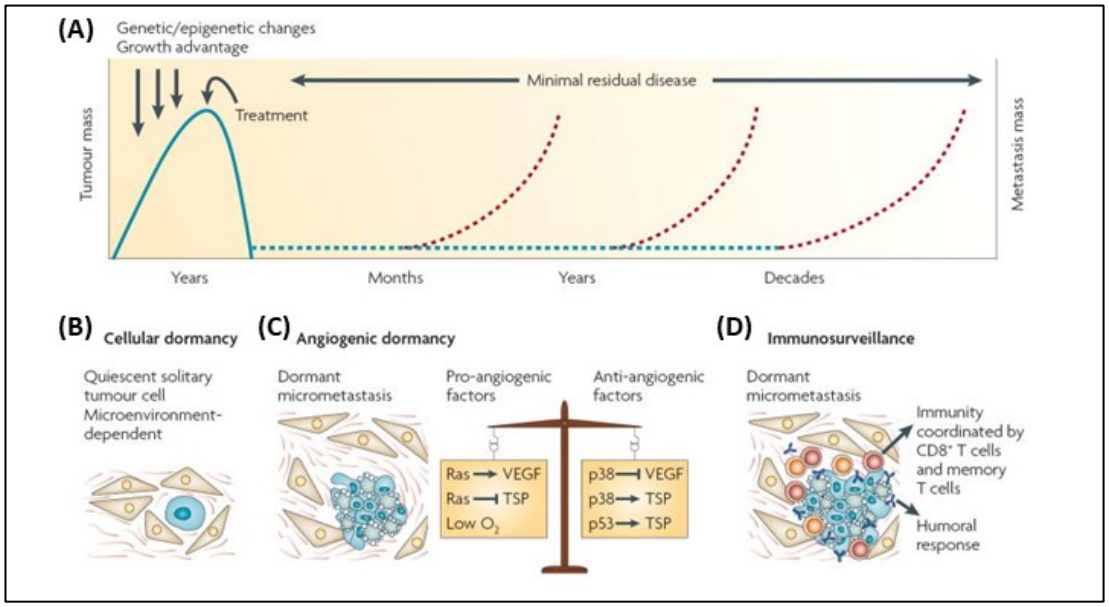


Figure 1. Mechanisms of cancer cell dormancy. (A) Cell that acquire genetic and epigenetic changes form a primary tumour (solid blue line), which regresses following treatment. A very small number of cancer cells that remain in the body after treatment (dashed blue line) and tumours can remerge again in a distant organ (dashed red line) months, years or even decades after remission. Cancer cell dormancy can manifest as a result of (B) Cellular dormancy, where cells enter G0–G1 arrest and are quiescent. (C) Angiogenic dormancy. Pro- and anti-angiogenic factors such as vascular endothelial growth factor (VEGF) and thrombospondin (TSP) can result in insufficient angiogenic potential. Oncogenic Ras can induce VEGF and repress TSP, whereas, the stress-activated kinase p38 and the tumour suppressor p53 can induce TSP or repress VEGF. (D) Active immunosurveillance coordinated

by to cytotoxic CD8+ T lymphocytes or anti-idiotypic antibodies against the B-cell receptor can prevent cancer cell proliferation. Adapted from (24).

1.2 Cancer stem cells

The cancer stem cell theory suggests that a small subpopulation of tumour cells (1-5%) described as cancer stem cells (CSCs), are the origin of most human tumours (Fig. 2A) (25). The CSC concept is based on the idea that the subpopulation of tumour cells (CSCs) have the ability to self-renew, differentiate and sustain the population of cancer cells in the same way that normal stem cells (NSCs) renew tissues and organs in the body (Fig. 2B). This concept stems from studies in acute myeloid leukaemias (26). A minority of human leukaemia cells expressing markers similar to normal hematopoietic stem cells have been shown to give rise to acute myeloid leukaemia in non-obese diabetic severe combined immune deficient (NOD SCID) mice whilst after transplantation, a large proportion of the population of tumour cells failed to engraft and initiate disease (Fig. 2B) (26,27). It has also been shown that cells which initiate leukaemias, have the ability to generate cells that lose their capacity to initiate disease, which recaptures the cellular heterogeneity of leukaemia and demonstrates a functional hierarchy (26). CSC or tumour-initiating activity has been illustrated in multiple tumour types including breast, lung and colon (25,28,29). In a breast cancer model, engrafted mesenchymal stem cells (MSCs) cause otherwise weakly metastatic human breast cancer cells to enhance their metastatic potential and generate more metastases supporting the theory that NSCs may generate CSCs (30).

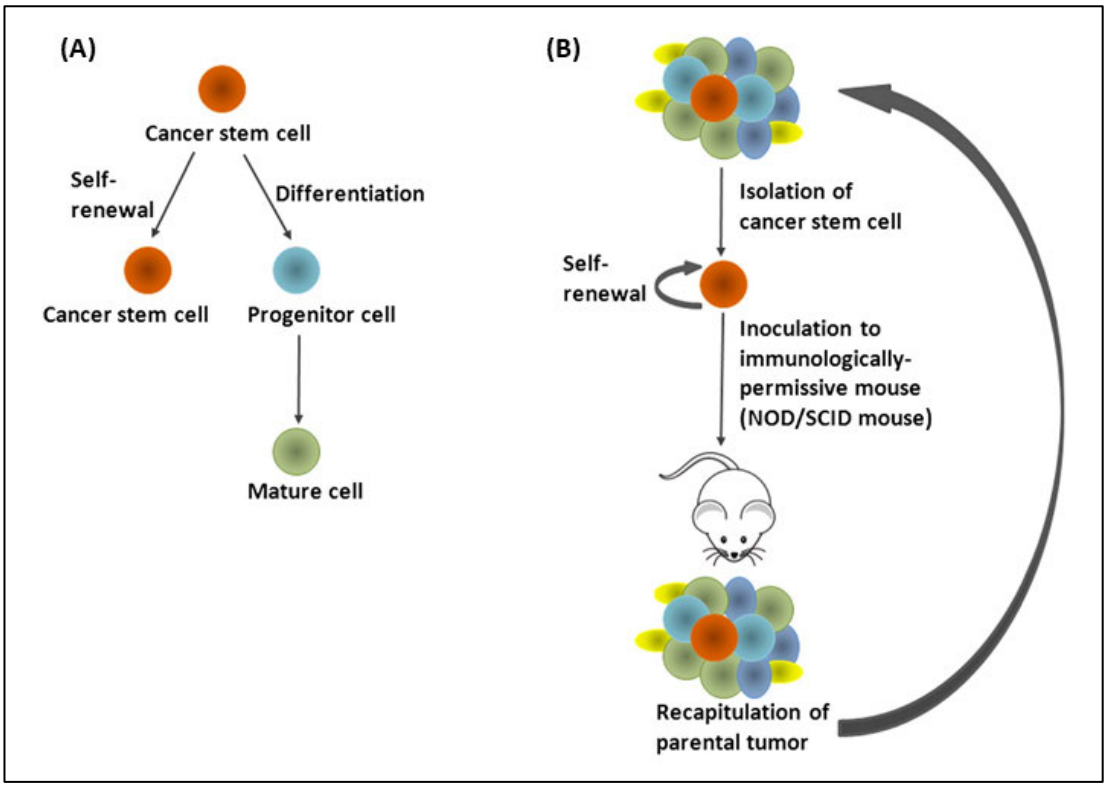


Figure 2. The cancer stem cell theory. (A) A subpopulation of tumour cells (CSCs) have the ability to self-renew and differentiate. (B) This population of CSCs has the capacity to generate a tumour across a number of serial passages in mice. Reproduced from (31).

The CSC theory assumes the population of CSCs are responsible for sustained tumour growth and that cancer stem cells are accountable for relapse following cancer treatment. CSCs may have increased drug and chemotherapy resistance compared to differentiated tumour cells and may also express anti-apoptotic genes unlike differentiated cells (32). Although surgery, radiation, chemotherapy and anti-cancer drugs may either eliminate the signs of cancer or shrink tumour size, if the cancer stem cells are not eradicated, the tumour may remerge or grow back and CSCs may also drive the formation of metastases at distant sites due to their self-renewal capabilities (33). Hence, metastatic breast cancer mortality also remains high due to the occurrence of therapy-resistant cancer cells (34).

1.2.1 Markers of breast cancer stem cells

Several markers that have been characterised for breast CSCs have high overlap with markers of normal mammary stem cells (MaSCs). The cell surface markers of breast CSCs have been determined through *in vivo* experiments in which human breast cancer cells are grown in immunocompromised mice. One study showed that the oncogenic subpopulation of cells expressed $CD24^-/CD44^+$ and only this minority of tumour cells could initiate new tumours (25). This population of cells were able to generate more $CD24^-/CD44^+$ cells as well as a population of non-oncogenic cells across a number of serial passages, demonstrating the ability to self-renew and also, generate a heterogeneous population of tumour cells (25).

Cells with the cell surface markers $CD24^+/CD29^h$ have been shown to expand in the mouse mammary tumour virus (MMTV)-Wnt-1 mouse model (35) and the Mouse mammary tumour virus-polyoma middle tumour-antigen (MMTV-PyMT) mouse model (36). Furthermore, cells with $CD24^+/CD49f^h$ markers were also identified as responsible for generating outgrowths following mammary fat pad transplantation (37). Breast cancer cell lines with the BRCA1 mutation have also been shown to have a subpopulation of cells with $CD24^+/CD29^h$ or $CD24^+/CD49f^h$ cell surface markers and these cells displayed amplified proliferation, colony forming capacity *in vitro*, and heightened oncogenic ability *in vivo* (38).

The markers $Lgr5^+$ and $Procr^+$ have been identified in $CD44^+$ breast CSC populations and these are also found on the cell surface of MaSCs (39–43). Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) and protein C receptor (PROCR) are target genes of the Wnt pathway, a pathway that is significant for initiation of mammary organogenesis, subsequent generation of the mammary gland and in breast tumorigenesis through regulation of stem cell self-renewal, expansion and differentiation (44,45). Overexpression of LGR5 in breast cancer is significantly linked to recurrence of breast malignancy and poor survival (42,46). By activating Wnt-β-

catenin signalling, LGR5 promotes breast CSC self-renewal and epithelial-mesenchymal transition (EMT) to promote carcinogenesis (Fig. 3) (42,46). EMT is a process which is important during development but has been linked with cancer progression (Fig. 4) (47). In EMT, basal epithelial cells undergo biochemical modifications that enable the cell to adopt a mesenchymal phenotype (47). Epithelial cells become motile and this leads to an invasive phenotype (47). Similarly, research has shown that PROCR expression also induces tumour formation and is important for breast CSC maintenance (48). Moreover, procr^{+} MaSCs have EMT signatures, which possibly suggests that procr^{+} MaSCs are precursors of breast CSCs (41).

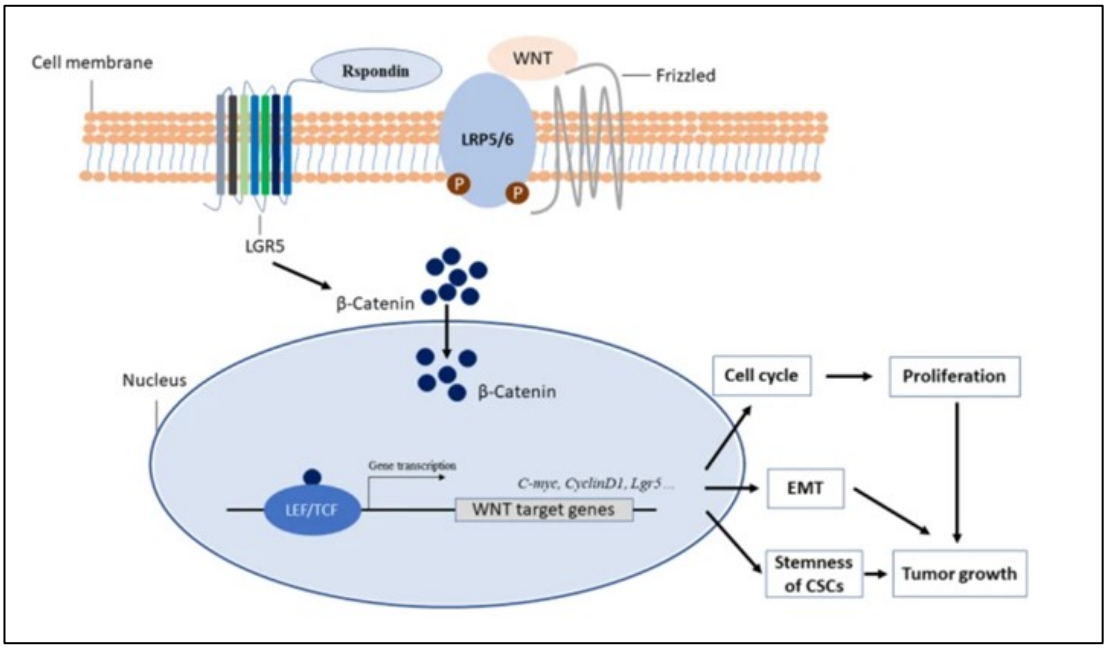


Figure 3. Leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) promotes breast cancer stem cell (CSC) self-renewal and epithelial-mesenchymal transition (EMT) to promote carcinogenesis by activating Wnt-β-catenin signalling. Lgr5 is activated by R-spondin and has been proposed to recruit the LRP-frizzled receptor complex. LRP5/6 are phosphorylated and the LRP-frizzled receptor complex binds to Wnt ligands. Wnt signalling leads to the accumulation of β-catenin, which is translocated to the nucleus and binds with transcription factors (including members of the T cell-factor (TCF) and lymphoid enhancer-binding factor (LEF) family) to induce expression of Wnt target genes (including C-myc, Cyclin D1, Lgr5). This promotes tumorigenesis by activation of the cell cycle and cell proliferation, EMT and maintenance of CSCs. Reproduced from (49).

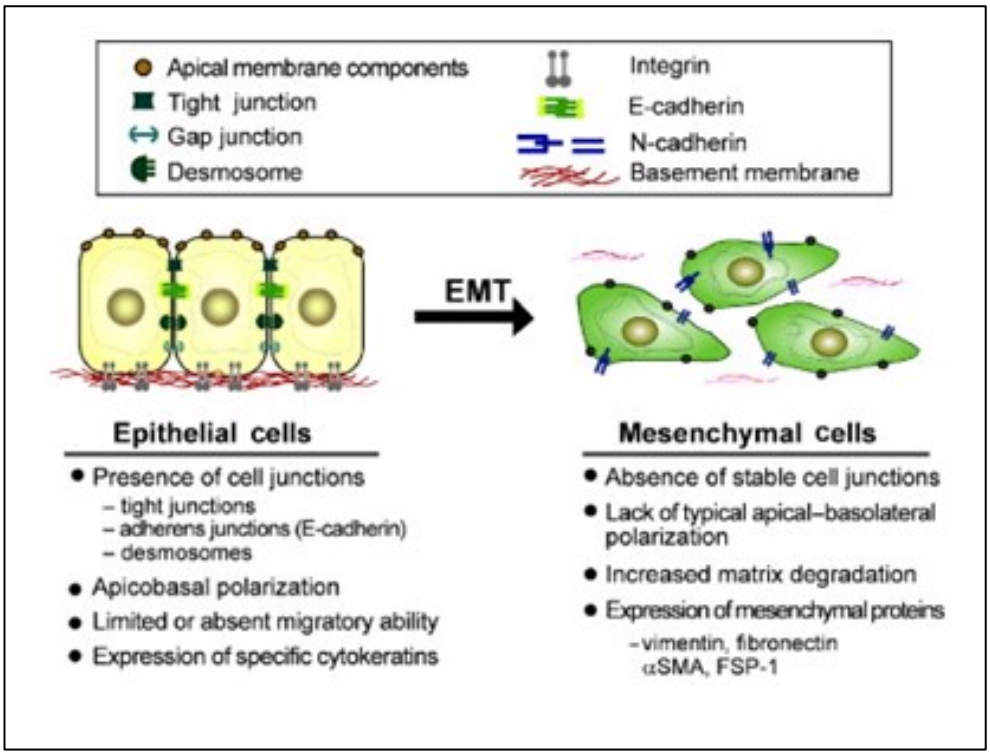


Figure 4. Epithelial and mesenchymal cell features. During epithelial-mesenchymal transition (EMT), epithelial cells lose their apical-basal polarity and connections to the basement membrane. The process is regulated by transcription factors including; ZEB, SNAIL and TWIST, which inhibit genes that are related to the epithelial state (47). Cells gain mesenchymal features including the expression of mesenchymal markers and display front-to-back polarity. Acquisition of focal adhesions and extensive actin cytoskeleton reorganisation results in increased migratory and invasive capacity. This is reversible process that occurs both during normal development and cancer progression. Reproduced from (50).

$ALDH^+$ activity has been identified as a stem cell marker in both MaSCs and breast CSCs and increased ALDH levels in breast malignancy is a predictor of poor patient outcome (51). Notably, a universal signature of CSC markers has not been clearly defined and there are many different mutational profiles across different subtypes of breast malignancies, which proposes that the CSC phenotype may be tumour-type dependent (52).

1.3 Metastasis

Metastasis describes the spread of cancer cells from the primary tumour to the regional lymph nodes, and distant organs within the body which consequently results in the formation of secondary tumours or metastases (Fig. 5) (53). Both molecular

changes in the tumour cells and the tumour environment enable cancer cells to detach from the primary tumour, invade local tissues and migrate into the tumour stroma (53,54). During local invasion, the basement membrane is broken down and tumour cells invade the surrounding ECM (55). Tumour cells then enter the blood circulation by intravasation to migrate to distant organs. Following entry into the blood circulation, the tumour cells are referred to as circulating tumour cells (CTCs). The CTCs must avoid immune destruction, endure mechanical stress and attach to endothelial cells in order to undergo subsequent extravasation and colonise distant organs (54,56). Tumour cells are termed disseminated tumour cells (DTCs) once settled at a distant site and may remain dormant as either single cells or micrometastases for several years before developing into macrometastases (57). The DTCs must establish a specialised microenvironment called the metastatic niche that permits angiogenesis and proliferation in order to form larger macrometastases (56). The metastatic niche is formed by a variety of different cell types and soluble factors secreted by the primary tumour and cancer associated fibroblasts. The ECM is also thought to be important in this context as it is frequently transformed in tumours and is essential for development patterning, tissue reorganisation as well as the stem cell niche (23,58). In addition to distal metastasis or hematogenous metastasis, which has been described here and is defined by the dissemination of the tumour cells via the blood circulation, the tumour cells may enter and spread through the lymphatic system and result in lymph node metastasis (59,60).

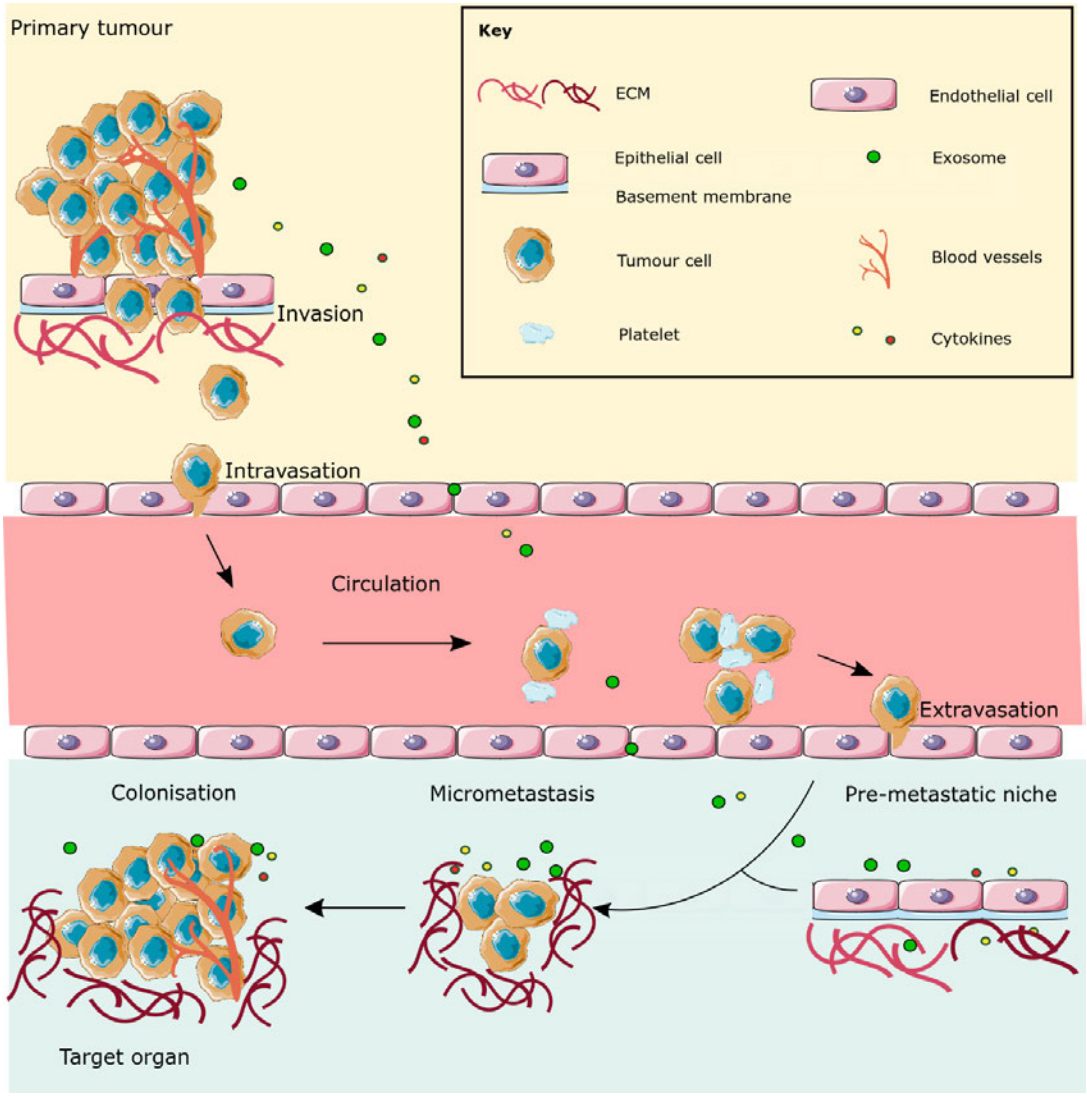


Figure 5. The stages of metastasis. Multiple steps lead to metastasis of tumour cells. Tumour cells firstly undergo local invasion whereby, the basement membrane is broken down and tumour cells migrate into the surrounding extracellular matrix (ECM) of adjacent tissues. Tumour cells then enter the circulation in a step termed intravasation and are known as circulating tumour cells (CTCs). The CTCs travel through the blood vessels coated in platelets as either single cells or clusters of cells. In order to spread successfully to distant organs, the CTCs must evade destruction by the immune systems and survive mechanical stress. Tumour cells then undergo subsequent extravasation and colonise the target organ by attaching to endothelial cells. Once settled in the metastatic organ, tumour cells are termed disseminated tumour cells (DTCs). It is essential that the DTCs survive the foreign environment and form a microenvironment that permits angiogenesis and proliferation in order to establish micrometastases and later, form large macrometastases. Created using Inkscape and Servier Medical Art.

1.3.1 Treating metastasis in breast cancer

Even though the mortality rate of breast cancer has substantially declined over the decades, increased survival of patients with cancer over time has leaned towards

patients with primary tumour growth and not those suffering from metastatic disease (19). It has been proposed that preventing metastasis may be a more effective strategy compared to targeting existing metastatic lesions. In order to reduce existing metastatic lesions, millions of tumour cells need to be targeted with radiation or a cytotoxic therapy, which results in cell death and tumour shrinkage. On the other hand, to prevent metastasis, fewer tumour cells need to be targeted and in addition to cytotoxic agents, cytostatic agents that inhibit tumour growth without direct cytotoxicity can be used (61).

Adjuvant therapy has shown some success for the treatment of metastasis in breast cancer. Examples include the use of tamoxifen or aromatase inhibitors that treat hormone receptor positive breast cancer (62,63). Adjuvant therapy using these agents has been shown to improve disease-free survival and overall survival in patients (62,63). In addition, continuing the use of adjuvant tamoxifen from five to ten years has been shown to approximately half the mortality rate of breast cancer patients (64) and prolonged use was also shown to decrease tumour recurrence (65). Mechanistically, tamoxifen has been hypothesised to influence the microenvironment of tumour cells to promote dormancy and prevent metastasis (65). The drug trastuzumab that is used for treatment of the HER2 subtype of cancer is another example of adjuvant therapy which has improved overall survival and significantly decreased cancer recurrence in breast cancer patients (66–68). However, although some patients may benefit in the short-term from adjuvant therapies, adjuvant treatment is not always effective due to differences in the biology of disseminated cancer cells and the primary tumour. The disseminated tumour cells are able to remain clinically asymptomatic as a result of dormancy (Fig. 1) and dormancy enables these cells to resist targeted therapy (24,69). Importantly, some studies have suggested that breast cancer cells disseminate from the breast to a distant organ 5-7 years before initial diagnosis (70). Therefore, it has been suggested that inducing or maintaining dormancy of DTCs could be potential therapeutic avenue to target metastasis (71). Additional therapeutic options that have been

proposed include targeting either DTC survival or the mechanisms of DTC drug resistance (71).

Aguirre-Ghiso, Bragado & Sosa (72) have proposed that DTC profiling should be trialled in the clinic. This would involve characterising the molecular and phenotypic profiles of DTCs to determine which and when patients might relapse and identify DTC-specific mechanisms to target. As patients with DTC-positive bone marrow frequently have worse prognosis compared to patients with DTC-negative bone marrow, Aguirre-Ghiso, Bragado & Sosa (72) proposed that DTC-negative bone marrow patients should be monitored for DTC appearance. Moreover, the authors suggested that DTCs from DTC-positive patients should be profiled for proliferative markers. If proliferation markers are found, patients should be targeted with conventional anti-proliferative treatments in combination with a dormancy-inducing therapies (Table 2). However, if the DTCs from DTC-positive patients display dormancy markers then dormancy-inducing therapies could be used alone to extend dormancy (Table 2). There are several drugs already in the clinic that could be suitable to induce dormancy in DTCs (Table 2). The DNA methylation inhibitor 5-azacytidine has been shown to downregulate expression of cell cycle G0 to G1 exit genes including DNMT1 and FOXM1 (73,74). This was demonstrated in breast cancer and leukaemia lines and genes such as RARB and CDKN1A (genes that are upregulated in p38-induced dormancy) were also upregulated (73,75). This suggests that demethylating agents combined with RARα and RARβ agonists are a possible therapy for inducing tumour cell dormancy (Table 2). Retinoic acid coupled with 5-azacytidine has also been found to induce a dormancy phenotype *in vivo* similar to bone marrow and p38-induced dormancy (73,75,76). HDAC inhibitors have also shown promise for inducing prolonged dormancy in uveal melanoma (77).

Table 2. DTC and niche markers and potential therapies for DTC-positive patients.

Patients with disseminated tumour cell (DTC)-positive bone marrow are considered to have a poor prognosis (72) and therefore, DTCs from these patients could be profiled to test for proliferation markers (e.g. upregulation of COCO, fibronectin, periostin (POSTN), transforming growth factor-β1 (TGFβ1) or phosphorylated ERK (P-ERK) and low expression of phosphorylated p38 (P-p38)) or dormancy markers (e.g. down-regulation of P-ERK, high expression of P-p38, TGFBR3, bone morphogenetic protein receptor type 2 (BMPR2), TGFβ2 or BMPs). If DTCs display proliferative markers, then DTCs could be targeted with conventional anti-proliferative drugs coupled with dormancy-inducing drugs. Conversely, if DTCs exhibit dormancy markers then dormancy-inducing drugs alone could be used. It is possible that expression profiles from DTCs might help to detect specific targets to eliminate DTCs. DTCs positive for interferon-γ (IFNγ) receptors may be sensitive to immune therapy, chloroquine may eliminate autophagic dormant DTCs, and SRC and MEK inhibitors may cause the eradication of DTCs (as reviewed in (71)).

Type of cancer	DTC and niche markers	Potential therapy	Potential patient outcome
Dormant DTC phenotype			
Prostate, breast and HNSCC	• DTC: TGFβR3 ↑, BMPR2 ↑, P-ERK ↓, P-p38 ↑ and GRP78 ↑ • Niche: TGFβ2 ↑, BMP4 and BMP7 ↑	• TGFβ2, BMP4 and BMP7 inducers or active biologicals • Chaperone and MEK–ERK inhibitors	Chronic asymptomatic MRD
Breast, glioblastoma, osteosarcoma and liposarcoma	DTC: POSTN ↓ and TSP receptors ↑	POSTN blockers, TSP and angiomotin inducers	Chronic asymptomatic MRD
Ovarian	DTC: ARHI ↑ and ATG genes ↑	Chloroquine	MRD eradication
Pancreatic	• DTC: IFNR ↑ and TNFR ↑ • Niche: active $CD4^+$ $CD8^+$ T cells	• IFNγ • TBVA-derived peptide vaccines, OX40 agonist	Chronic asymptomatic MRD
Active DTC phenotype			
Breast	DTC: EDG2 ↑, P-Src ↑, P-ERK ↑ and P-p38 ↓	AZD0530/6244	MRD eradication
HNSCC and breast	• DTC: P-ERK ↑ and P-p38 ↓ • Niche: fibronectin ↑, COCO ↑ and TGFβ1 ↑	• 5-Aza-C, HDAC inhibitors, anti-RARα and anti-RARβ retinoids • Anti-β1, anti-α5β1 or anti-TGFβ1 mAbs	Chronic asymptomatic MRD

5-Aza-C, 5-azacytidine; ATG, autophagy-related gene; BMP, bone morphogenetic progenitor; HDAC, histone deacetylase; HNSCC, head and neck squamous cell carcinoma; mAbs, monoclonal antibodies; MRD, minimal residual disease; TBVA, tumour-associated blood vessel antigen; TGFβ, transforming growth factor-β; TNFR, tumour necrosis factor receptor; TSP, thrombospondin.

Reproduced from (71).

As previously described earlier in this chapter, tumours cells are thought to lie dormant until a cue within their microenvironment triggers proliferation to resume (Fig. 1) (78) and the term colonisation describes the outgrowth of disseminated tumour cells (Fig. 5). Some research into targeting metastasis has surrounded the colonisation stage as it is considered a promising window for treatment. By targeting metastatic colonisation, colonising tumour cells are either directly destroyed or their

state of dormancy is extended. There are several drugs that are either under development or in clinical trials that aim to target breast cancer metastasis and specifically, target the colonisation stage. These include inhibitors against a family of non-receptor tyrosine kinases called Src. Src activates a number of proteins called receptor tyrosine kinases, which have roles in motility survival, proliferation and angiogenesis (79). A role for Src in colonisation has been suggested as Src gene expression has been associated with onset of bone metastasis in all breast cancer sub-types (80). In addition, mice studies where Src expression has been depleted or mice have been treated with Src inhibitors has prevented the formation of breast cancer metastases in a range of different breast cancer mouse models (80–85).

Drugs which block the ligand binding motifs of the integrin family have been shown to inhibit cancer cell invasion and metastasis in mouse models (86,87). The integrins are a family of heterodimeric cell surface transmembrane receptors formed by an alpha and beta subunit (Fig. 6). They participate in bidirectional signalling across the plasma membrane and regulate processes such as adhesion, migration and invasion (their role will be described in more detail later in this chapter, in 1.4.1.) (88,89). Cilengitide, a drug that targets αvβ3 and αvβ5 integrins may be suitable to treat bone metastasis as a mouse xenograft model demonstrated that osteolytic lesions form slower in mice treated with the drug (90). Bone metastasis was also inhibited by a small molecule antagonist of αvβ3, S247, in a mouse model where mice received an intracardiac injection of MDA-MB-435 cells (91).

Inhibitors of Focal Adhesion Kinase (FAK) are also under investigation as metastasis inhibitors. FAK is a non-receptor kinase that localises to focal adhesions and has important cell functions that are primarily through regulation of the cytoskeleton and through integrin mediated signalling pathways (92). Inhibition of FAK either through disruption of FAK expression or by using inhibitors, has been shown to prevent breast cancer metastasis to the lung and bone in a number of mouse models (93–96).

Fibrosis inhibitors have also shown potential for the treatment of metastasis. Fibrosis is a disease caused by the thickening of connective tissues due to inflammation and excessive production of ECM that is caused by the activation of myofibroblasts (97). Lysophosphatidic acid (LPA) induces fibrosis through binding to a G-protein coupled receptor and antagonists have been developed to target this receptor (98). Inhibition of the receptor for LPA, using an antagonist called Debio-0719, has also been shown to suppress breast cancer metastasis and induce tumour cell dormancy (99). Debio-0719 reduced breast cancer metastasis to the liver and lungs in a 4T1 spontaneous metastasis model and similar results were also reported using an experimental pulmonary metastasis model using MDA-MB-231 cells (99).

1.4 The Kindlin family

The Kindlin family consists of three four-point-one, ezrin, radixin, moesin (FERM) domain-containing focal adhesion proteins, Kindlin 1-3, that share high sequence identity (Table 3) (100). Kindlin-1 and Kindlin-2 share the highest sequence similarity as they evolved from Kindlin-3 (Table 3) (100). Kindlin-1 is the founding member of the Kindlin family of proteins and was named after a loss-of-function mutation in the gene encoding Kindlin-1, *FERMT1,* was shown to cause Kindler Syndrome (101). Kindler syndrome is a rare, autosomal recessive genodermatosis which causes skin atrophy, blistering, photosensitivity, hyper or hypo-pigmentation, increased light sensitivity and an enhanced risk of developing aggressive squamous cell carcinoma (SCC) (100,102,103).

Following the finding that mutations in the *FERMT1* gene cause Kindler syndrome (101), mutations in the *FERMT3* gene that encodes Kindlin-3 were found to cause leukocyte adhesion deficiency (104). Kindlin-2 is not associated with inherited disease but loss of Kindlin-2 expression has been shown to cause embryonic lethality in mice, (105,106). All three of the Kindlin isoforms have been associated with acquired disease including; kidney fibrosis (107), human glomerular disease (108),

atherosclerosis (109), polycystic ovary syndrome (110), neuropathic pain (111), heart failure (112), osteoporosis (113) and immune deficiency (114). In addition, both increased and decreased expression of Kindlin proteins have also been shown to promote tumorigenesis in a range of different tumour types (reviewed in (115)).

Table 3. Sequence similarity between the Kindlin family of proteins.

Proteins	Sequence Similarity (%)
Kindlin-1 and Kindlin-2	62
Kindlin-1 and Kindlin-3	49
Kindlin-2 and Kindlin-3	53
Kindlin-1 and Talin-1	28

Sources: (100,116)

The Kindlin proteins are differentially expressed in tissues, although, some cells may express more than one isoform. Kindlin-2 is ubiquitously expressed with the exception of haematopoietic cells and is predominantly enriched in skeletal and smooth muscle cells (Table 3) (117). It is also the only Kindlin isoform expressed in embryonic stem cells (Table 3) (106,117). Kindlin-1 is predominantly found in epithelial cells such as the skin, intestine and kidney whereas Kindlin-3 is primarily expressed in haematopoietic cells (100,117,118). Homologue-specific functions for each of the Kindlins in multiple signalling pathways have been identified as a result of differential expression in tissues and subcellular compartments (Table 4) (117,119). The Kindlins have conserved amino acid stretches that are presumed to facilitate the common functions of the family, whereas the variable regions of the Kindlin sequences are thought to enable the unique functions (100).

Table 4. Expression of the Kindlin family in different cell types and tissues.

Organ, Tissue or Cell Type	Kindlin-1	Kindlin-2	Kindlin-3

Bladder	+	+	-
Brain	-	+	-
Colon	+	+	-
Embryonic stem cell	-	+	-
Endothelial cell	-	+	+
Epithelial cell	+	+	-
Fibroblast	-	+	-
Heart	-	+	-
Kidney	+	+	-
Leukocyte	-	-	+
Liver	-	+	-
Lung	-	+	+
Lymph node	-	+	+
Skeletal muscle	-	+	-
Skin	+	+	-
Small intestine	+	+	-
Spleen	-	+	+
Stomach	+	+	-
Thymus	+	+	+

Sources: (117,119–121)

1.4.1 Kindlin is a regulator of integrin activation

A number of studies have shown that loss of the Kindlin isoforms results in defective integrin activation and consequently, impaired integrin regulated cellular functions (18,29,31). The integrins are a family of heterodimeric transmembrane receptors, which are important for cell adhesion - they connect the cytoskeleton with the ECM (122,123). The integrins are composed of an α and β subunit and at least 18 α and 8 β subunits are known (124). The α and β subunits have large extracellular domains that bind the ECM, a single membrane-spanning helix and a cytoplasmic tail (Fig. 6) (123). The cytoplasmic tail binds intracellular signalling molecules and adaptor proteins, which connects integirns to numerous signalling pathways and provides a link to the actin cytoskeleton (125).

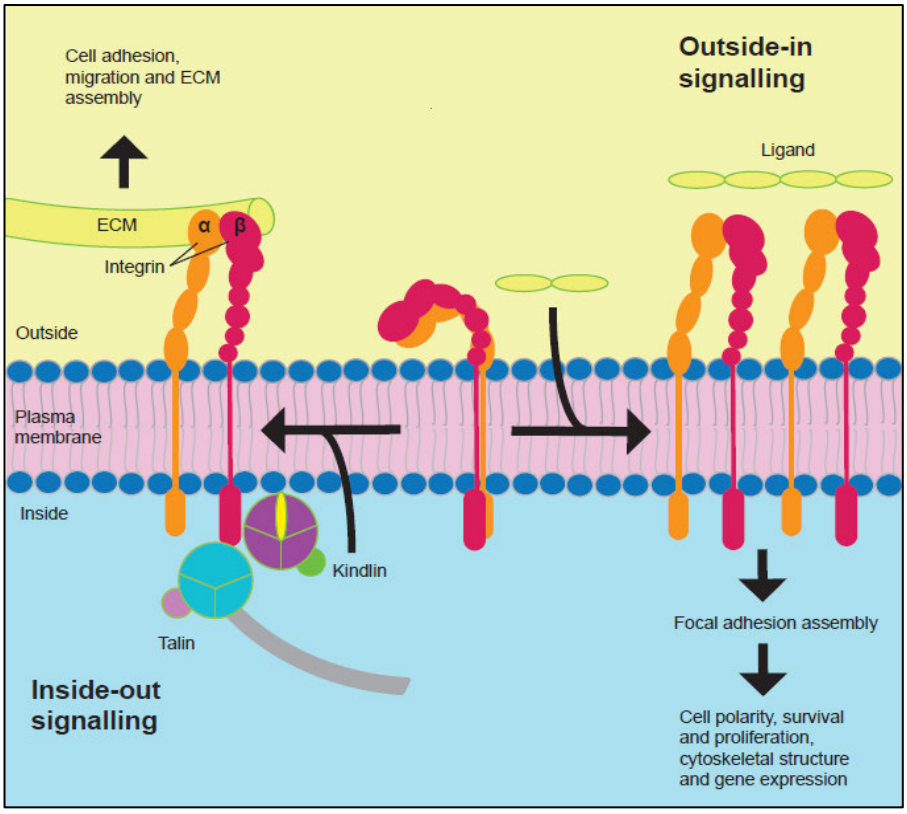


Figure 6. Kindlin and Talin mediate integrin activation and control integrin bidirectional signalling across the plasma membrane. Integrins partake in both inside-out an outside-in signalling across the plasma membrane, which enables many cellular processes such as ECM assembly, cell adhesion, migration and proliferation to occur. Kindlins cooperate with talin intracellularly to activate integrins by binding β-integrin tails via their four-point-one, ezrin, radixin, moesin (FERM) domains. Upon Kindlin and Talin binding to β-integrin tails, 'inside-out signalling' is initiated (left). The integrins change conformation from a bent inactive conformation (centre) into an extended, active high affinity state (left) that has high affinity for extracellular matrix (ECM) ligands. Integrins also can also facilitate

'outside-in signalling'. ECM ligands such as fibronectin bind to the extracellular heads of integrins (right) and stimulate conformational changes which leads to integrin clustering. This triggers focal adhesion complex assembly of which Kindlins and Talin are key components of and leads to intracellular signalling. (Created using Adobe Illustrator CC 2018)

Integrin activation describes the transformation of integrins from an inactive low affinity state to an active high affinity state and facilitates bidirectional signalling across the plasma membrane. Bidirectional signalling is important for diverse cellular processes including; ECM assembly, cell adhesion modulation, migration, proliferation and survival (Fig. 6) (reviewed in (126)). The integrins participate in 'outside-in signalling' where ligands bind to integrin extracellular domains, stimulating integrin clustering and increasing integrin-ligand binding avidity (124,127). Subsequently, this stimulates assembly of large macromolecular complexes called focal adhesion (FA) complexes and activates numerous signalling pathways inside cells (126). The integrins also participate in 'inside-out signalling' where intracellular proteins such as Talin and Kindlin bind to β-integrin tails to facilitate integrin activation (128,129).

A study in *Caenorhabditis Elegans* was the first study to propose the importance of the Kindlin-integrin interaction (129). A homozygous null mutation in *unc-112,* the worm ortholog of *FERMT2* caused abnormal assembly of dense bodies and M lines in muscle cell basal membranes, which was similar to abnormalities observed for mutations in worm orthologues of β-integrin and an ECM component, perlecan (129). UNC-112 was shown to colocalise with the worm ortholog of β-integrin (PAT-3) and was required for localisation to the muscle membrane (129). In 2003, mutations in the *FERMT1* gene, which encodes a human homolog of UNC-112, Kindlin-1, were shown to cause KS (100). However, studies that demonstrated that Kindlin was essential in integrin activation came much later, in 2008, when null mutations in *FERMT3* were shown to decrease integrin activation in platelets, that still expressed Talin (a protein previously thought to be the only mediator of integrin activation) (128,130). Correspondingly, depletion of Kindlin-2 was shown to impair β-integrin activation in podocytes (100).

1.4.2 Structure and function of Kindlin

The Kindlins do not exhibit catalytic activity and have been identified as adaptor proteins, which bind to integrins (Fig. 6) and mediate additional protein-protein interactions via their FERM domains (Fig. 7) (reviewed in (131)). FERM domains are found in other proteins, which function to connect the plasma membrane of the cell with the cytoskeleton such as FAK and Talin (132). Unlike most FERM domains, which have subdomains F1-F3, the FERM domains of the Kindlin family have five subdomains; F0-F3 and a pleckstrin homology (PH) domain that transects the F2 domain (Fig. 7) (101,133). The PH domain is a unique feature of the Kindlin FERM domain and binds to lipids (101). The PH domain targets the Kindlin proteins to the plasma membrane by binding to phosphoinositides and localises the Kindlin proteins to focal adhesions for integrin activation (134,135).

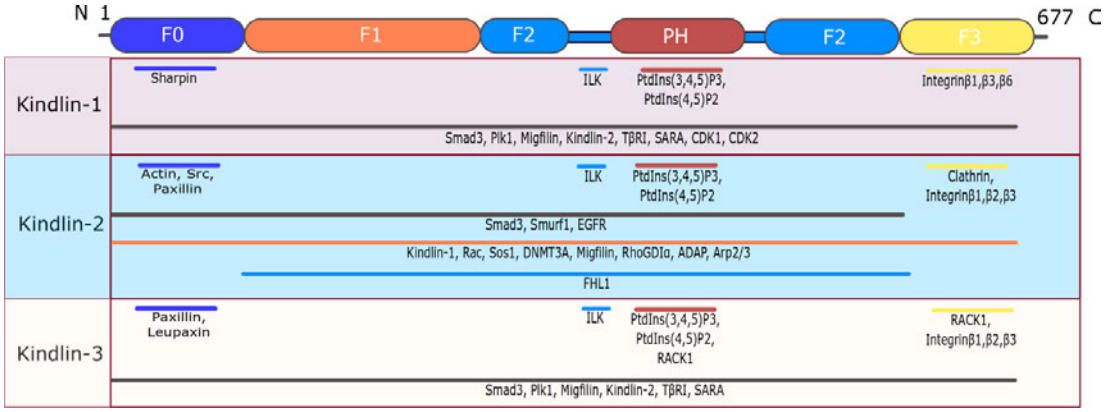


Figure 7. Schematic of Kindlin FERM domain organisation and known Kindlin-binding proteins. The FERM domain consists of five subdomains: F0-F3 and a Pleckstrin homology (PH) domain. The coloured bars indicate the position where each of the known Kindlin-binding proteins bind Kindlin 1-3. Black lines indicate an unknown binding region. Figure created using Inkscape. Sources: Binding partners were previously reviewed in (115,131,136) and sources (137–139) were used to adapt the summary of the current literature surrounding the Kindlin interactors.

It was previously believed that Talin was the only regulator of integrin activation but later studies showed that Kindlin was also required (128,129,140). Kindlin and Talin

share a high level of sequence similarity (Table 3) and there is close sequence similarity between their FERM domains (Table 5) (133,141). Both Kindlin and Talin FERM domains have an F0 subdomain, which is important for effective integrin activation and has phospholipid binding sites (Fig. 8) (133,141). Mutation of F0 in Kindlin-2 disrupts membrane binding and prevents cooperative activation of integrin αIIbβ3 with Talin (142). Moreover, Chinese Hamster Ovary (CHO) cells that lack a Kindlin-1 F0 domain have impaired integrin activation (133). Kindlins 1-3 have similar F0 subdomain structures and their F0 subdomains contain a ubiquitin-like fold similar to Talin (142).

Table 5. Kindlin and Talin FERM domains share sequence similarity

Domains	Sequence Similarity (%)
F0: Kindlin and Talin	36
F1: Kindlin and Talin	55
F2: Kindlin and Talin	50
F3: Kindlin and talin	54

Sources: (133,141)

The F1 subdomain also has phospholipid binding sites (Fig. 8). The F1 subdomain has an unstructured loop with a membrane targeting motif, which is involved in targeting Kindlin to the focal adhesions (143). Notably, the loop in the F1 domain of Kindlin is at the same position as Talin (between strands of a ubiquitin-like fold) but the loop in the Kindlin F1 domain is much longer than the loop in the Talin F1 subdomain and there is little sequence similarity (143). The F1 loop is conserved between the Kindlin isoforms and species which, suggests an important role (143). The F1 loop has been to shown to enable cooperative αIIbβ3 integrin activation of Kindlin and Talin as

Kindlin-1 and -2 were unable to activate αIIbβ3 integrin with loss of this loop with presence of functional Talin and conversely, Kindlin was unable to activate αIIbβ3 integrin if Talin lacked the F1 loop (143).

Both Kindlin and Talin directly interact with β-integrin cytoplasmic tails via a phosphotyrosine-binding (PTB)-fold located in the F3 subdomain (101,106,144,145). Kindlin-1 binds to β cytoplasmic tails via a conserved tryptophan and distal NxxY motif, whereas Talin binds via a proximal NxxY motif (Fig. 8) (146,147). Studies have suggested that Kindlin and Talin cooperatively regulate integrin activation as Talin without Kindlin or Kindlin without Talin is not sufficient to trigger integrin activation (Fig. 6) (104,106,148,149).

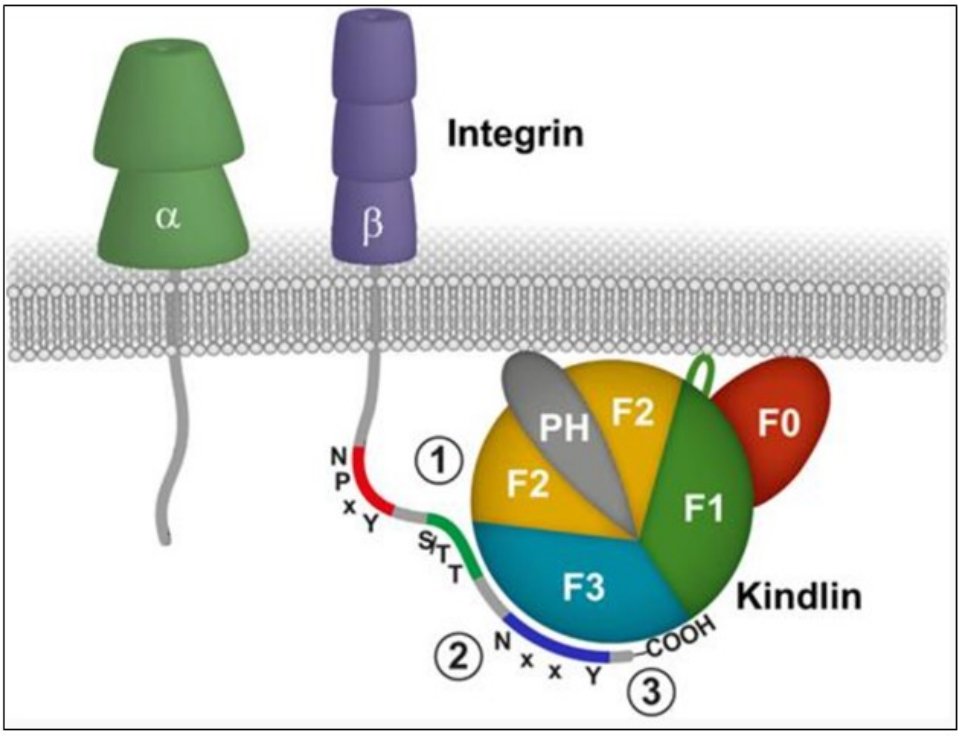


Figure 8. Kindlin-1-integrin and cell membrane binding. The Kindlin proteins bind to the β-integrin cytoplasmic tail via the FERM F3 subdomain through a serine and/or threonine motif (1), the membrane distal NxxY motif (2) and the C-terminus (3). The Pleckstrin homology (PH) domain of the Kindlin FERM domain binds to phosphoinositides and the positively charged regions of the F0 and F1 subdomains bind to the negatively charged membrane. Reproduced from (131).

The F2 subdomain is fundamental to the differential functions of Kindlin-2 and Kindlin-3 (150). In particular, the C-terminal of the F2 subdomain supported localisation of Kindlin-2 but not Kindlin-3 to FAs (150). Functionally, Kindlin-3 deviates from Kindlin-1 and -2, which both have a key role for ECM adhesion through their interaction with integrins at FAs (151,152). Even though Kindlins 1-3 bind integrins, Kindlin-3 cannot localise to FAs, including when Kindlin-3 is present in adherent cells (130,145,148). Kindlin-3 does localise to podosomes, which are specialised transient adhesion structures in hematopoietic cells but only Kindlin-1 and -2 have been shown to localise to and function at FAs (117,119). The presence of a variable glycine-rich sequence in the C-terminal of the F2 subdomain in Kindlin-3 is partly responsible for differential subcellular localisation and the loss of targeting of Kindlin-3 to FAs (150). It has been postulated that this insertion may encode an extra site for protein binding or a flexible linker that inhibits its recruitment to FAs (150). This functionally may be important for the role of Kindlin-3 in haematopoietic cells (150). As the only Kindlin family member found in haematopoietic cells, it has been hypothesised that the loss of permanent association of Kindlin-3 at FAs might be required for Kindlin-3 to enable rapid adhesion in these cells (150).

The Kindlins are differentially distributed when expressed in the same cell types, which suggests different roles within cells. For example, in endothelial cells, Kindlin-3 was found at extending membrane areas in the cell whilst Kindlin-2 was found at the FAs (119). Kindlin-1 and -2 have also been shown to localise to different cell adhesion sites and so even though there are overlapping functions of this family of proteins, the Kindlin proteins have developed additional specific functional adaptations in different cells and tissues (117). Deletion of Kindlin-1 in a mouse model resulted in loss of epithelial cell adhesion to the basal membrane demonstrating that Kindlin-2 is unable to compensate for loss of Kindlin-1 (149). Furthermore, Kindlin-1 has been shown to have specific role in cell adhesion to the fibronectin-rich ECM (153,154).

Kindlin-2 has been found predominantly at FAs in epithelial cells, keratinocytes and breast cancer cells where its recruitment to FAs is connected to its role in cell spreading (153–155). In these studies Kindlin-1 does not compensate for cells deficient in Kindlin-2 as Kindlin-2 deficient cells have impaired spreading (153–155). Some of the functional differences of the Kindlin family have been associated with differential binding to β-integrin subunits, which connect the Kindlins with different cellular signalling pathways. Whilst all three of the Kindlin isoforms can bind to β1 and β3 integrins, Kindlin-1 is the only isoform that binds β6 and it does not bind to β2 integrin like Kindlin-2 and -3 (Fig. 7) (106,130,145,148,149,153,156–158). Binding of Kindlin-1 to the β6-integrin tails is particularly important in disease, including; KS as epithelial cells lack Kindlin-1 and SCC as there are high levels of αvβ6 integrin and low levels of Kindlin-1 (153,156). Kindlin-2 cannot compensate for lack of Kindlin-1 interaction with αvβ6 and less activation of this integrin leads to disease phenotypes (156).

As well as functional differences, there are also functional similarities between the Kindlin family. In breast cancer cells, Kindlin-1 is predominantly found at the perinuclear region and Kindlin-2 was found in the cell periphery suggesting that Kindlin-2 is predominantly located at FAs (155). Kindlin-1 was shown to relocate with Kindlin-2 inactivation and may compensate for the functions of Kindlin-2 as simultaneous loss of Kindlin-1 and Kindlin-2 had severe effects on cell shapes, size and migration consistent with data in keratinocytes and fibroblasts (154,159), whereas loss of either Kindlin-1 or Kindlin-2 alone had little effect, emphasising redundancy (155).

1.4.3 Kindlin binding partners

In addition to binding to integrin, several other binding partners have been identified for the Kindlin isoforms (Fig. 7) and these interactions relate to the different Kindlin functions (as reviewed in (115,131,136)). Related to its role in integrin activation,

Kindlin-1 has been shown to bind to Sharpin and suppress β1-integrin activation in CHO cells (Fig. 9A) (138) and unsurprisingly, the Kindlins have been found to bind to other focal adhesion proteins. All three of the Kindlin isoforms bind to the focal adhesion protein, Integrin-Linked Kinase (ILK) (Fig. 9B) (160). Research has demonstrated that Kindlin-2 binds to the ILK complex through a linker region located within its F2 PH subdomain and this, in addition to integrin-binding (via its F3 subdomain), is necessary for optimal Kindlin-2 accumulation at FAs (Fig. 3) (160). Notably, Kindlin-3 does not localise at FAs and was found to bind to ILK differently when compared to Kindlin-2 (160). ILK, like the Kindlin family of proteins is required for effective integrin signalling; mice embryos that do not express ILK are unable to polarise their epiblast and fail to form cavities, which leads to death at the peri-implantation stage (161). ILK, although initially thought to have kinase activity, lacks key catalytic residues in its kinase domain and has therefore been coined a pseudokinase (162). The interaction of Kindlin with ILK will covered in more detail in chapter 4.

Kindlin-1 and Kindlin-2 have both also been reported to bind to Migfilin (Fig. 9B) (118,163), a focal adhesion protein that regulates cell shape and is important in cell spreading (164). Interaction of Kindlin and Migfilin is thought to be important for Migfilin recruitment to FAs, as well as localisation and mobility (163).

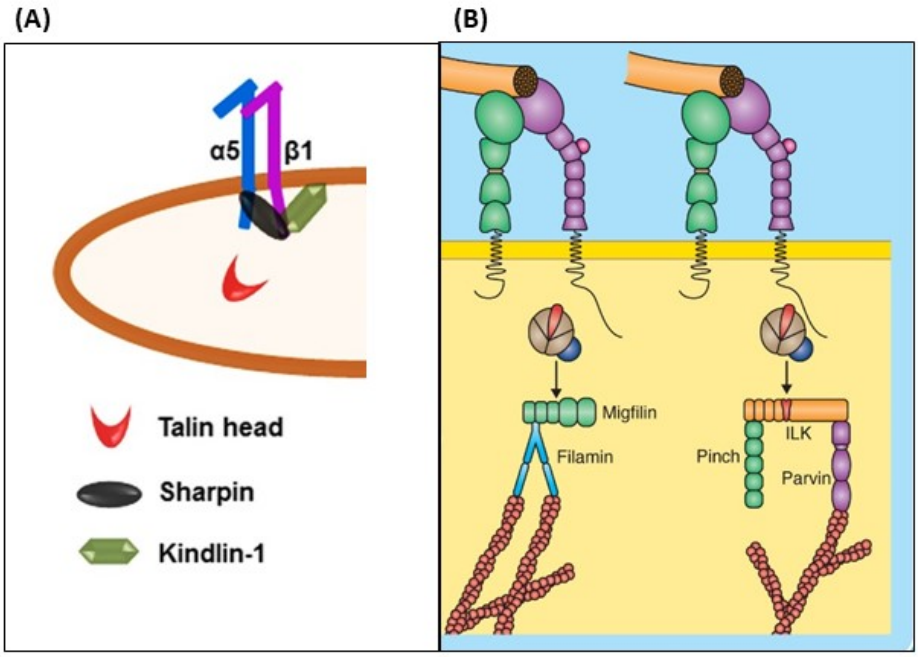


Figure 9. Kindlin-1 binds focal adhesion proteins. (A) Sharpin directly binds to Kindlin-1 and β1 integrin cytoplasmic tails and competitively inhibits the Talin head. This leads to negative regulation of α5β1 activation in Chinese Hamster Ovary cells. (B) Upon integrin activation, Kindlin binds to Migfilin (left) and Integrin-Linked Kinase (ILK) (right), which connects the integrin to the cytoskeleton and enables cell spreading. Adapted from (121,138).

Co-immunoprecipitation experiments have confirmed binding of Kindlin-1 with Smad anchor for receptor activation (SARA), Smad3 and to the TGF-β receptor I (TβRI) in colorectal cancer cells and has linked Kindlin-1 to transforming growth factor β (TGF-β) signalling (Fig. 10A) (165). The TGF-β pathway normally has tumour suppressor functions as it is involved in regulation of the cell cycle and apoptosis but in cancer, its activation has been shown to promote metastasis and chemoresistance by inducing EMT (166,167). The interaction of Kindlin-1 with SARA, Smad3 and TβRI was found to mediate Smad3 phosphorylation and nuclear translocation. This resulted the activation of TGF-β/Smad3 signalling pathway, which promoted EMT (165).

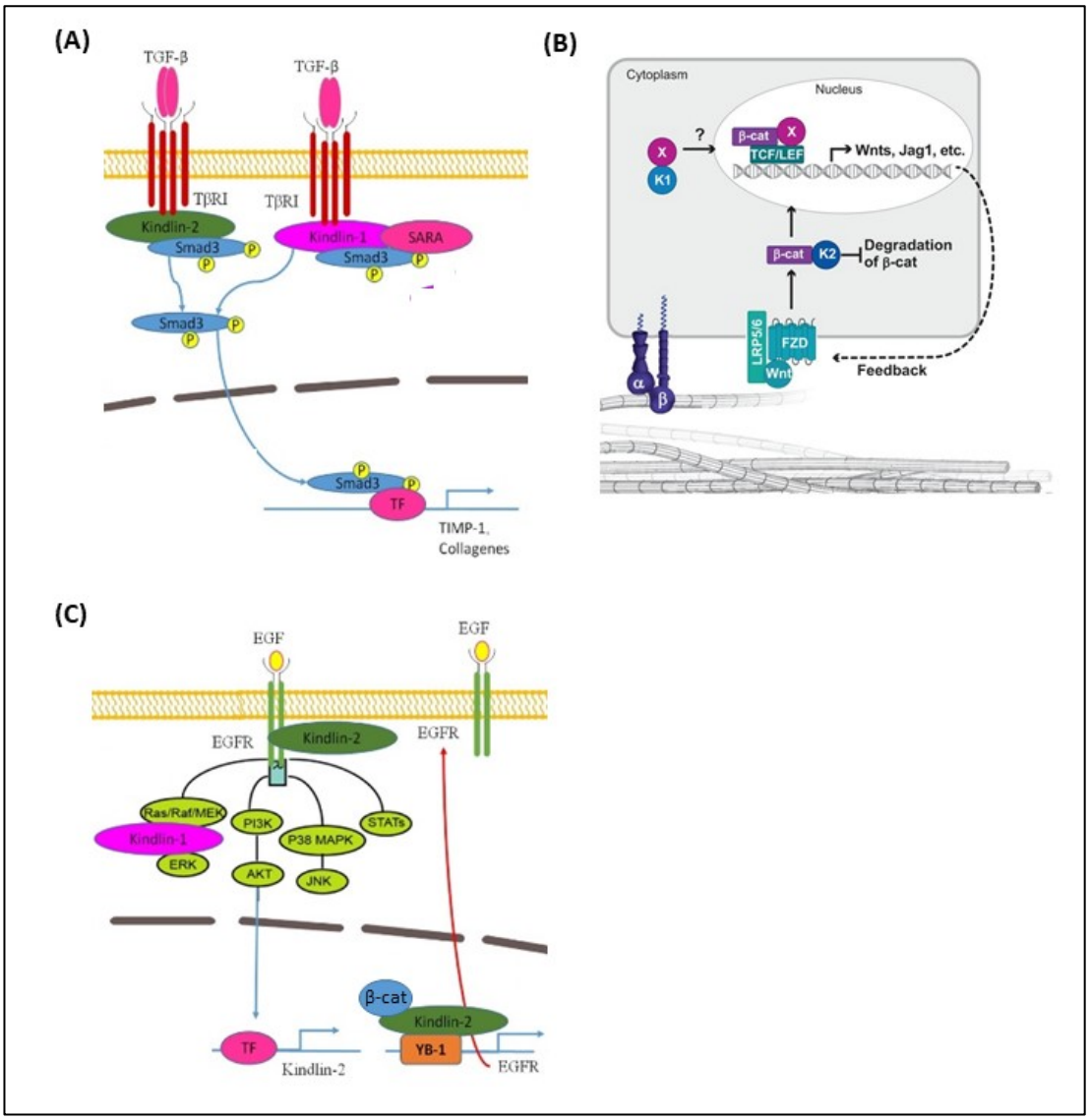


Figure 10. The Kindlins regulate a number of different signalling pathways. (A) Kindlin and TGFβ signalling. Kindlin-2 binds directly to TGFβ receptor I (TβRI) and promotes Smad3 phosphorylation and TGFβ signalling. Kindlin-1 has been shown to form a complex with TβRI, Smad3 and SARA to regulate TGFβ signalling. (B) Kindlins and Wnt signalling. Kindlin-1 regulates Wnt independent of integrin and may transcriptionally inhibit Wnt signalling by retaining transcriptional cofactors in the cytoplasm. Ablation of Kindlin-1 increases Wnt ligand transcription, induction of canonical Wnt signalling and upregulation of Wnt target genes. Kindlin-2 directly binds β-catenin, which inhibits its degradation. Kindlin-2 forms a complex with active β-catenin and T-cell factor 4 (TCF4), to form a transcriptional complex that binds to the promoter of Wnt target genes and stimulates gene expression. (C) Kindlin-1 has been shown to be required for full activation of ERK signalling and this protect cells from oxidative. Kindlin-2 expression is upregulated by EGFR signalling and Kindlin-2 also forms a complex with Y-box binding protein-1 (YB-1) and β-catenin, and binds EGFR promoter to increase transcription of EGFR. Adapted from (115,131).

In keratinocytes, Kindlin-1 has also been shown to bind to the cyclin-dependent kinases (CDKs), CDK1 and CDK2, which are regulators of cell cycle progression (137).

This interaction was shown to be important to regulate cell cycle arrest in response to oxidative stress (137). Kindlin-1 also interacts with Polio-like kinase (Plk-1) in breast cancer cells and this is important for its role in mitosis (briefly discussed later in this chapter (1.4.5) and in more detail in chapter 4) (168).

1.4.4 Integrin independent roles

The role of the Kindlin proteins in integrin activation is most established, however, the Kindlins also have integrin-independent roles, which have more recently been recognised and these roles are still being discovered. Studies have explored integrin-independent functions of the Kindlin family by using mutants that contain mutations to the QW motif that binds integrin, which is located in the F3 subdomain of the FERM domain. This has linked the Kindlins to a number of signalling pathways independent of integrin binding and these include roles in the Wnt, TGF-β and Epidermal growth factor receptor (EGFR) pathways.

Kindlin-1 and Kindlin-2 have been shown to regulate Wnt signalling, a signalling pathway that regulates a number of cellular processes including angiogenesis and cell growth (156,169,170). The transcription of Wnt ligands and receptors was enhanced in keratinocytes from floxed Kindlin-1 mice and it has been hypothesised that mechanistically, Kindlin-1 may transcriptionally inhibit Wnt signalling by retaining transcriptional cofactors in the cytoplasm (Fig. 10B) (156). Re-expression of an integrin binding-deficient Kindlin-1 mutant demonstrated that the mechanism which Kindlin-1 inhibits Wnt signalling does not require integrin binding (171). Conversely, Kindlin-2 has been shown to enhance Wnt signalling in the nucleus and contains a nuclear localisation signal (NLS) that tags the protein for nuclear import (117). Kindlin-2 promotes Wnt signalling in breast cancer cells by binding active β-catenin and T-cell factor 4 (TCF4), to form a transcriptional complex that binds to the promoter of Wnt target genes and stimulates gene expression (Fig. 10B) (169,170).

Interestingly, an NLS has not yet been identified for Kindlins 1 and 3 but they have been shown to localise to the nucleus in keratinocytes (Kindlin-1) and human umbilical vein endothelial cells (Kindlin-3) (118,119,156). Therefore, it is possible that all three Kindlin isoforms have integrin independent roles within the nucleus, which are yet to be recognised.

In addition to Wnt, Kindlin-2 has an integrin independent role in the TGF-β signalling pathway, a pathway that has been linked to disease including cancer when deregulated (166). Kindlin-2 has also been shown to mediate TβRI and Smad3 binding in kidney tubular epithelial cells (Fig. 10A) (107). Reduction of Kindlin-2 expression suppressed the interaction of TβRI with Smad3, preventing TGF-β/Smad3 signalling and expression of target genes whilst overexpression of Kindlin-2 amplified TGF-β-induced Smad3 activation (107). The regulation of TGF-β1-induced Smad3 activation by Kindlin-2 is integrin-independent as expression of a Kindlin-2 mutant that cannot bind to β1 integrin did not rescue the phenotype and knockdown of β1 did not recapitulate the effect that reduction of Kindlin-2 expression had (107). In accordance, in pancreatic ductal adenocarcinoma (PDAC) cells, a positive feedback loop occurs with Kindlin-2 and TGF-β expression that regulates PDAC progression (172). Kindlin-2 has been identified as a TGF-β target gene in PDAC and therefore, increased TGF-β signalling results in increased Kindlin-2 protein expression (172). Conversely, TβRI is upregulated by Kindlin-2, which is important for TGF-β signalling (172). HoxB9 and E-cadherin were downregulated as a result of TGF-β1-induced Kindlin-2 expression, which promoted PDAC progression (172).

The EGFR signalling pathway is also promoted by Kindlin-2 and Kindlin-1 (Fig. 10C). Kindlin-2 forms a complex with Y-box binding protein-1 (YB-1) and β-catenin, which binds to the EGFR promoter to increase transcription and this also promotes expression of YB-1 and β-catenin (Fig. 10C) (173). In breast cancer, Kindlin-2 has also been shown to bind the EGFR kinase domain and prevent ubiquitination and degradation of EGFR, which induces tumour cell migration (174).

In addition to roles in cellular signalling pathways, Kindlin-2 has been shown to control clathrin-dependent trafficking of endothelial cell-surface enzymes by directly interacting with clathrin via the F3 subdomain of its FERM domain (175). This interaction was shown to be important for haemostasis as loss of Kindlin-2 in a mouse model caused increased expression of adenosine triphosphate diphosphohydrolase and ecto-5'-nucelotidase at the surface of endothelial cells preventing platelet aggregation (175).

1.4.5 Roles of Kindlin-1 in Kindler syndrome and the skin

Specific cellular roles have been identified through depletion of Kindlin-1 expression or by using Kindlin-1 mutants that have impaired function. Loss of Kindlin-1 has been shown to impair integrin activation *in vitro* (101) and *in vivo;* in KS skin (176) and the intestinal epithelium of mice with deletion of Kindlin-1 (149). Defective integrin activation resulted in reduced adhesion to the integrin ligands; fibronectin and laminin 322 in cultivated KS keratinocytes (176–178) and caused significant adhesion defects in intestinal epithelial cells of mice with deletion of Kindlin-1 (149). Adhesion defects are likely to be connected to the skin abnormalities in KS patients such as atrophy, fragility and the tissue separation found within or below the basal keratinocyte layer (177). In addition to lethal intestinal epithelial dysfunction due to epithelial detachment, mice with deletion of Kindlin-1, had a severe inflammatory reaction, which resembled ulcerative colitis that is observed in KS patients (149). Kindlin-1 has also been shown to regulate the inflammatory response in the skin. In response to cellular stress, KS-deficient keratinocytes were found to increase the expression of the cytokines: IL-20, IL-24, TGF-β2, IL1F5, PDGFB, and CTGF in the dermis (179). Progressive fibrosis of the dermis follows inflammation in KS and activation of TGF-β, CTGF and IL-6 are thought to causal factors (180). These cytokines are important for extracellular matrix synthesis by fibroblasts and have been linked to fibrotic diseases (180).

As well as adhesion, Kindlin has also been shown to regulate a number of other cellular roles in keratinocytes. Kindlin-1 was shown to regulate proliferation, polarity and motility (177) and defective β1-integrin function was connected to abnormal cell morphology, focal adhesion assembly and cell migration (178). In addition, cell spreading defects have been observed in cultured KS keratinocytes (178) and in human immortalised keratinocytes with knockdown of Kindlin-1 (101). Kindlin-1 has also been identified as important for regulation of mitosis although, this was reported in breast cancer cells not keratinocytes (168). Kindlin-1 is phosphorylated by Plk-1 at the centrosomes of mitotic cells and regulates mitotic spindle formation and orientation (168).

Research has also suggested that Kindlin-1 is linked to regulation of stem cells in the skin. Loss of Kindlin-1 has been shown to reduce stem cell markers in KS skin and lower the colony-forming capacity of keratinocytes (176). Clonogenic activity is a sensitive indicator of undifferentiated stem cells suggesting that Kindlin-1 is involved in regulation of stem cells (176). Other studies found that KS keratinocytes exhibit precocious senescence, decreased clonogenic activity and reduced stem cell markers (181). This was linked to Kindlin-1 deficiency as a similar phenotype was observed in normal keratinocytes with Kindkin-1 targeting siRNA (181). In addition, Kindlin-1 has been shown to regulate the proliferation and differentiation of cutaneous epithelial stem cells through two different mechanisms (156). Kindin-1 was shown to activate the release of TGF-β via αvβ6 integrin (Fig. 11) and also inhibit Wnt-β-catenin signalling through regulation of the expression of Wnt ligand via a mechanism that is independent of integrin (156). It has been suggested that KS skin atrophy may be caused by loss of the regulation of cell stemness by Kindlin-1.

KS patients have an increased risk of developing squamous cell carcinoma (SCC), however, little is known about the mechanisms that lead to increased risk of KS patients developing SCC. Mice with an epidermal deletion of Kindlin-1 have

accelerated tumour onset and greater tumour burden, which is thought to be connected to the important role that Kindlin-1 has in regulating skin homeostasis (156).

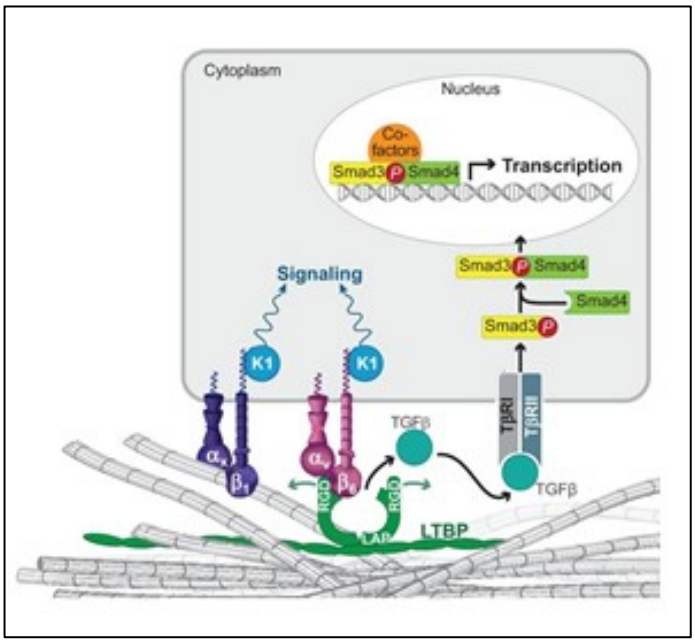


Figure 11. Kindlin-1 activates TGFβ signalling by mechanism dependent on integrin and regulate the proliferation and differentiation of cutaneous epithelial stem cells. Activation of αvβ6 integrin through Kindlin-2 binding, causes binding of αvβ6 integrin to the RD motif of the latency-associated peptide (LAP). LAP is associated with the latent TGFβ-binding protein (LTBP), and stimulates release of TGFβ, which binds to the transforming growth factor beta receptors I and II (TβRI and TβRII). Consequently, Smad3 is phosphorylated and forms a complex with Smad4. This complex is translocated to the nucleus and together with cofactors, stimulates the transcription of TGFβ target genes.

1.4.6 Kindlin-1 in cancer progression

Deregulation of Kindlin expression has been reported in cancers from different organs and abnormal expression has been shown to inhibit and promote different tumour types. Kindlin-1 expression has been shown to inhibit progression of oesophageal cancer (182) and lung cancer (183) but promote the progression of pancreatic cancer (184), colorectal cancer (165) and early pulmonary breast cancer metastasis (185,186). Mutation of Kindlin-1 or reduction of Kindlin-1 expression has been shown to increase the probability of skin cancer as previously discussed (187).

Kindlin-1 has also been reported as a prognostic factor for overall and disease free survival in hepatocellular carcinoma (188) and osteosarcoma (189).

1.4.6.1 The Kindlins and breast cancer

The Kindlin-2 isoform was the first Kindlin isoform to be associated with breast cancer and was linked to increased breast cancer invasion (190). Subsequently, there have been conflicting studies with reports of both upregulation and downregulation of Kindlin-2 linked to breast tumorigenesis (164,170,191–193). Interestingly, although Kindlin-3 is not expressed in epithelial cells, a study demonstrated a tumour suppressive role for *FERMT3* in breast cancer (194). *FERMT3* mRNA expression was downregulated in breast cancer tissues compared to normal breast tissues and knockdown of Kindlin-3 in mice was also shown to enhance breast cancer metastasis (194). Azorin *et al* (155) identified that Kindlin-3 localisation is confined to infiltrating immune cells of normal breast tissue and found that Kindlin-3 expression was highly correlated with immune marker levels in breast tumours. Upregulation of Kindlin-3 was shown to correlate with ER status and molecular subtype with HER2 and TNBC tumours containing the highest levels of Kindlin-3 transcripts (155).

A potential role for Kindlin-1 in primary tumour growth has also been suggested for Kindlin-1. Kindlin-1 overexpression has been reported in human breast tumours that were compared to normal breast tissues (155). In addition, the gene encoding Kindlin-1, *FERMT1*, was identified in a six gene signature predictive of breast cancer metastasising to the lungs and this signature was significantly linked to the triple-negative basal-like subtype (195,196). Clinical data in human breast tumours supported this finding as increased expression of Kindlin-1 was significantly associated with breast cancer that metastasises to the lungs (186). Furthermore, breast tumours that expressed high levels of *FERMT1* transcripts were identified as predominantly hormone receptor negative, with the highest levels of Kindlin-1 expression identified in the triple-negative subgroup (155). Importantly, this has

suggested that Kindlin-1 could be a predictive marker for the identification of breast cancer patients that are more at risk of development of metastatic disease.

Little is known mechanistically about the role of Kindlin-1 in breast cancer lung metastasis although it has been reported that Kindlin-1 may control breast cancer invasion and metastasis by regulating TGFβ signalling and initiating EMT (186). Sin et al. (186) showed that silencing of Kindlin-1 reduced tumour growth and prevented lung metastasis in an orthotopic mouse model. Kindlin-1-depleted primary tumours had increased levels of E-cadherin compared to tumours that expressed Kindlin-1 (186). As loss of E-cadherin in cancer cells leads to activation of EMT transcription factors and has been shown to promote metastatic dissemination (197), decreased E-cadherin expression in Kindlin-1 expressing tumours was associated with EMT. Regulation of EMT by Kindlin-1 was associated with activation of the growth regulatory protein, TGFβ and Sin *et al.* (186) showed increased Smad-2 and Smad-3 activation, and upregulated expression of TGFβ target genes in the presence of Kindlin-1. Genes that were upregulated by Kindlin-1 included: connective tissue growth factor, endothelin 1, early growth response 1, TGFβ2 and matrix metalloproteinase 9, which are known to promote tumorigenesis (186).

Using the MMTV-PyMT-driven mouse model of mammary tumourigenesis, recent work by the Brunton group has shown that loss of Kindlin-1 significantly delayed tumour onset and reduced lung metastasis (185). Additionally, loss of Kindlin-1 reduced metastatic colonisation in experimental metastases assays using Kindlin-1 deficient cells. Mechanistically, Kindlin-1 enhanced metastatic growth in an integrin-dependent manner through integrin-dependent adhesion to endothelial cells in the metastatic niche. Furthermore, loss of β1-integrin decreased the outgrowth of micrometastases suggesting that Kindlin-1 may also have a role in outgrowth of micrometastases.

Proteomic analysis showed that Kindlin-1 regulated the secretion of a number of factors associated with metastasis including tenascin-C (185). Tenascin-C is a large hexameric ECM glycoprotein protein that has a multimodular structure enabling the protein to bind a number of diverse ligands including: fibronectin (198), periostin (199), integrins (200), and a range of soluble growth factors such as vascular endothelial growth factor (VEGF), TGF-β and fibroblast growth factor (FGF) (201). Tenascin-C is abundantly expressed in embryos at sites of morphogenesis (202), however, little is found in the adult, where it is restricted to tissues bearing high tensile stress and at sites of inflammation or tissue injury (203). Additionally, it is most prominently expressed in stem cell niches in the adult (204,205), at sites of EMT and is concentrated in the stroma of solid tumours (206). Particular interest in the involvement of tenascin-C in breast cancer stemmed from research which identified tenascin-C in a gene set that mediates breast cancer metastasis to the lung (207). Cancer cell-derived tenascin-C is important in the ECM of the metastatic niche and enables breast cancer cells to colonise the lungs through positive regulation of stem-cell signalling pathways (205). Regulation of the Notch and Wnt pathways by tenascin-C was shown to support breast CSC fitness and promote micrometastatic outgrowth (205). Bioinformatic analysis by the Brunton group found a positive correlation between *TNC* and *FERMT1* expression in MDA-MB-231 breast cancer cell line subpopulations predisposed to lung metastasis and in primary breast tumours associated with lung metastasis (185). This further suggests that Kindlin-1 has a role in regulation of early pulmonary metastasis of breast cancer through regulation of tenascin-C.

1.5 Hypothesis and project aims

Pulmonary metastatic breast cancer presents a major clinical challenge: patients with pulmonary breast cancer metastasis have a median survival rate of less than 2 years after diagnosis (208). Studies have demonstrated that Kindlin-1 is a mediator of pulmonary breast cancer metastasis and that its expression may also impact primary tumour growth (Fig. 12) (185,186,207,209). Silencing of Kindlin-1 has been shown to significantly reduce mammary primary tumour growth and prevent lung metastasis in mice (186), which suggests that targeting Kindlin-1 could present an effective strategy for targeting both primary tumour growth and metastasis. Translating these findings to the clinic provides a challenge as Kindlin-1 is an adaptor protein that does not exhibit catalytic activity. Therefore, an understanding of the mechanisms by which Kindlin-1 promotes breast cancer is imperative to uncover targets that may be exploited in order to treat breast cancer.

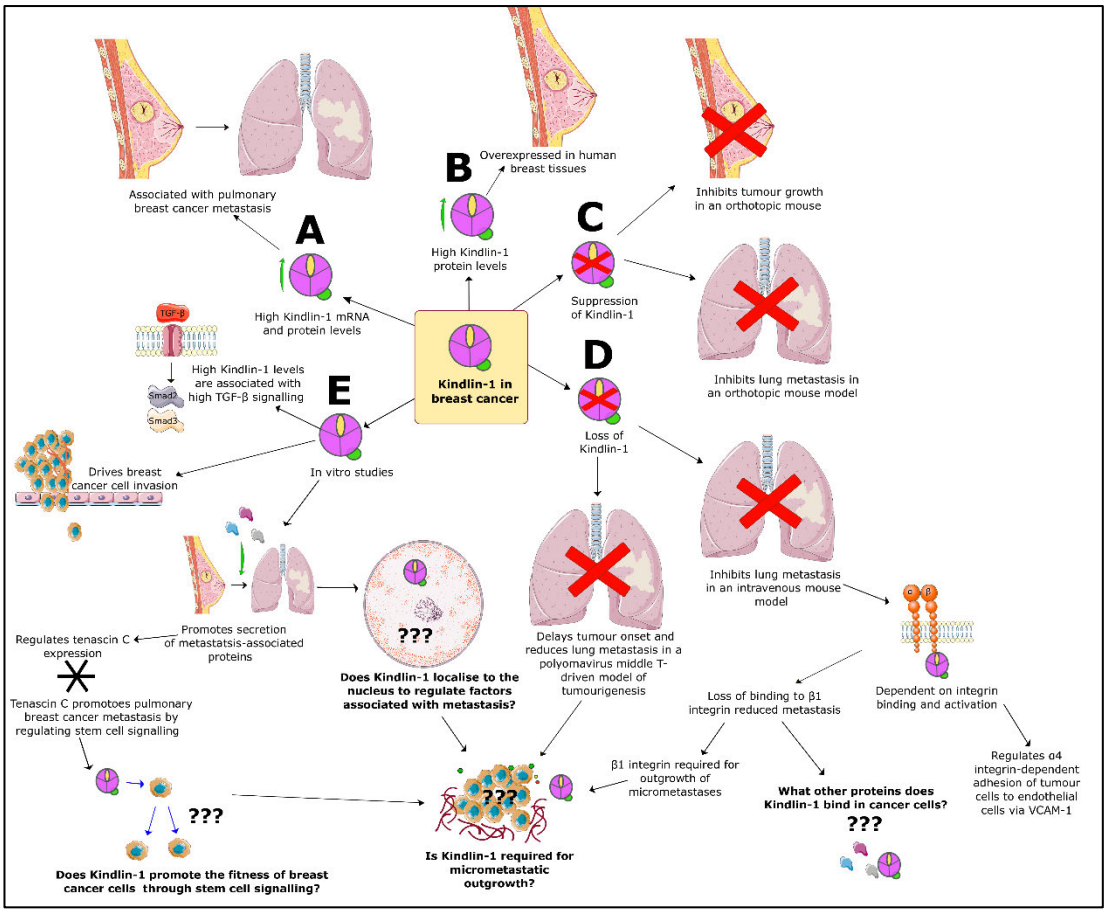


Figure 12. The role of Kindlin-1 in Breast Cancer. (A) The first studies connecting Kindlin-1 to breast cancer suggested a role for Kindlin-1 in the promotion pulmonary breast cancer metastasis (195,196). High levels of *FERMT1* transcripts and high Kindlin-1 protein levels were confirmed in breast tumours metastasising to the lungs (155,186). (B) Furthermore, when compared with normal breast tissue, breast tumour tissues have higher Kindlin-1 protein levels suggesting a potential role for Kindlin-1 in primary tumour growth (155). (C) Disruption of Kindlin-1 expression has been shown to prevent primary tumour growth and metastasis in an orthotopic mouse model of breast cancer (186). Primary tumours were found to have increased levels of E-cadherin with loss of Kindlin-1 and therefore, Kindlin-1 was suggested to promote EMT and tumour dissemination (186).
(D) Metastatic burden is reduced in a genetically modified mouse model of Kindlin-1 loss and an experimental metastasis mouse model of Kindlin-1 loss (185). Mechanistically, Kindlin-1 regulates α4 integrin-dependent adhesion of tumour cells to endothelial cells via VCAM-1 and activates β1-integrin to promote metastasis (185). As β1-integrin is required for outgrowth of micrometastases, this raised the question: is Kindlin-1 required for micrometastatic outgrowth? And in addition to integrin, what does Kindlin-1 bind to promote tumorigenesis?
(E) *In vitro* studies have shown that ectopic expression of Kindlin-1 increases proliferation (186), clonogenicity (186) and breast cancer cell invasion (155,186). High Kindlin-1 levels have been associated Kindlin-1 with TGF-β signalling, and activation of TGF-β was suggested to induce EMT (186). Kindlin-1 was also shown to promote the secretion of metastasis-associated proteins and regulate the expression of a protein called tenascin-C (185), which has been shown to support pulmonary micrometastatic outgrowth in breast cancer (205). Tenascin-C supports breast cancer cell fitness through its role in the regulation of stem cell signalling pathways (205), which leads to the question: does Kindlin-1 promote the fitness of breast cancer cells through stem cell signalling? And does Kindlin-1 localise to the nucleus to regulate factors associated with metastasis?

From the existing literature on Kindlin-1, which is demonstrated in Figure 12, the following hypothesis was formed:

Kindlin-1 regulates micrometastatic outgrowth by 1.) Supporting the fitness of breast cancer cells through regulation of stem cell signalling, and 2.) Localising to the nucleus of cancer cells to regulate factors associated with metastasis in breast cancer cells.

The aims of this project were to:

- Determine if Kindlin-1 regulates outgrowth of micrometastases by developing and characterising a pulmonary metastatic mouse model of human Kindlin-1 loss using human MDA-MB-231 cells (with inducible shRNA Kindlin-1).
- Establish if Kindlin-1 affects breast cancer stem cell activity by using the *in vitro* mammosphere assay in mouse Met-1 and human MDA-MB-231 cells (with inducible shRNA Kindlin-1).
- Identify binding partners of Kindlin-1 in different breast cancer cell models using proteomic technologies to define differential Kindlin-1 binding partners

in Met-1 cells and metastatic and non-metastatic variants of MDA-MB-231 cells.

- Explore a nuclear role for Kindlin-1 in breast cancer. Establish if nuclear Kindlin-1 has an important role in breast cancer cells by developing a Kindlin-1 mutant that cannot localise to the nucleus of mouse Met-1 cells and investigate if this impacts tumour growth *in vivo*.

Chapter 2 Materials and Methods

2.1 Cell culture

2.1.1 Cell lines and maintenance

Murine Met-1 Clone A1 Kindlin-1 Null (Kin1-Null), Kindlin-1 WT (Kin1-WT) and Kindlin-1 AA (Kin1-AA) cells and the Met-1 Clone 3B Kindlin-1 Null cell line used to generate a Kindlin-1 NLS (Kin1-NLS) cells as described in 2.1.4 were previously generated by the Brunton Group (185). Murine SCC Kindlin-1 Null (Kin1- Null), Kindlin-1 WT (Kin1-WT) and Kindlin-1 AA (Kin1-AA) cells were obtained from I. Stavrou (ECRC, Edinburgh) (210). Human MDA-MB-231 cells (parental and the lung metastatic variant, 4173) were obtained from B. Qian (QMRI, Edinburgh). Human MDA-MB-231 cells used for generation of the Kindlin-1 shRNA cell line were obtained from ATCC). Cell lines were cultured in Dulbecco's Modified Medium (DMEM) high glucose (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Gibco by Life Sciences). Met-1 and SCC cells were also supplemented with hygromycin B (200 µg/mL) (Invitrogen). The cell lines were maintained in a humidified incubation at 37 °C with 5% CO2. Cell lines were mycoplasma tested every month and were used within three months of recovery from frozen.

2.1.2 Generation of MDA-MB-231 inducible Kindlin-1 knockdown cell line

MDA-MB-231 cells were transfected with a doxycycline inducible TRIPZ *FERMT1* shRNA vector (shRNA Kin1) (Fig. 9A) and a TRIPZ non-targeting (NT) shRNA vector (for use as a negative control) as described previously by Dodd (211). Doxycycline (dox) (1 µg/mL) (Sigma Aldrich) was added to cells in order to induce transcription of the shRNA and knockdown the *FERMT1* gene. Knockdown of *FERMT1* expression was confirmed by western blot and real-time quantitative reverse transcription PCR (RT-qPCR) analysis (Fig. 9C-D).

2.1.3 Generation of Kindlin-1 knockout in MDA-MB-231 lung metastatic cell line

MDA-MB-231 cells were infected with a double nickase CRISPR/Cas9 knockout plasmid (Santa Cruz; sc-412553-NIC) or a control double nickase plasmid (Santa Cruz; sc-437281) (for use as a negative control) according to manufacturer's protocol except X-tremeGENE HP DNA Transfection Reagent was used as the transfection reagent (Roche; XTG360-RO) and opti-MEM reduced serum medium (ThermoFisher Scientific; 31985062) used as the transfection medium.

2.1.4 Generation of a Kindlin-1 nuclear localisation mutant cell line

The pWZL-Kindlin-1 WT vector previously generated by the Brunton group (185) was used to generate the pWZL-Kindlin-1 NLS mutant using the Q5® Site-Directed Mutagenesis Kit (New England BioLabs; E0554) as per manufacturers protocol. The primers used for mutagenesis are listed in Table 6. Plasmids containing mutations to the Kindlin-1 sequence were sequenced after each transformation before proceeding using the primers in Table 7. A retroviral infection protocol was used to infect the mutant Kindlin-1 NLS vectors into Met-1 3B Kindlin-1 Null cells previously generated by the Brunton group. The pWZL-Kindlin-1 WT and an empty pWZL vector were also infected into Met-1 3B Kindlin-1 Null cells. Phoenix ECO cells were seeded into 10cm plates 24 hours prior to transfection. A plasmid (10μg) and opti-MEM (0.5mL) reduced serum media mix was made and incubated at room temperature for 10 minutes. A lipofectamine mastermix was also made containing 20μL Lipofectamine 2000 (ThermoFisher Scientific; 11668030) and 0.5mL opti-MEM (ThermoFisher Scientific; 31985062) for each sample and incubated at room temperature for 10 minutes. Both mastermixes were combined and incubated for 15 minutes at room temperature. The media of Phoenix ECO cells was replaced with 5mL opti-MEM and the cells were transfected with lipofectamine/DNA mixes dropwise whilst swirling. The media was removed after 6 hours and replaced with DMEM 10% FBS. The media of Phoenix cells was replaced with DMEM 20% FBS (Viral induction media) after 24 hours (day 2) and Met-1 Kindlin-1 Null cells seeded into 10cm dishes. After 24 hours

(day 3) the media was harvested from the phoenix ECO cells and passed through a 0.45μM filter and added to Met-1 Kindlin-1 Null plates with polybrene transfection reagent (Sigma-Aldrich; TR-1003-G) at a concentration of 1:2000. Harvesting of the virus was repeated a second time the following day. The infected cells were left to grow until 50-60% confluency before selecting with puromcyin (100μg/mL). Cells were supplemented with fresh antibiotic every two days and passaged once confluency was reached. Western blot analysis was used to confirm that plasmids were successfully introduced into the Met-1 Kindlin-1 Null cell line and nuclear fractionation performed followed by western blot to confirm that Kindlin-1 NLS mutants were successful (Fig. 49C).

Table 6. Mutagenesis primers used to generate Kindlin-1 nuclear localisation mutants

Name (F/R)	Oligo (Uppercase = target-specific primer)	Tm	Ta *
R131A_F	GCTGAATATCgcaAGACCAGAAGAAC	57°C	58°C
R131A_R	ACTTTGCAGATATCAGCG	59°C	
R132A_F	GAATATCAGAgcaCCAGAAGAACTTTC	55°C	56°C
R132A_R	AGCACTTTGCAGATATCAG	59°C	
K131/132A_F	GCTGAATATCgcagcaCCAGAAGAACTTTCATTG	56°C	57°C
K131/132A_R	ACTTTGCAGATATCAGCG	59°C	
K147/148A_F	TGACTACTGTgccgcgAAAAAAAAGAAAGAAAAAAATAG	56°C	56°C
K147/148A_R	CTAGAAGGCTTTAACAATG	55°C	
K149/150A_F	CTGTGCCGCGgccgccAAGAAAGAAAAAAATAG	60°C	61°C
K149/150A_R	TAGTCACTAGAAGGCTTTAAC	59°C	
K151/152A_F	CGCGGCCGCCgccgccGAAAAAAATAG	57°C	59°C
K151/152A_R	GCACAGTAGTCACTAGAAG	60°C	

Table 7. Sequencing primers for mutagenesis

Primer Description	Sequence
Kindlin-1 NLS sequencing forward	CTTCGCCTGCCCAATGCC
Kindlin-1 NLS sequencing reverse	CCAAACCAGGTCATAGTG

2.2 Animals

Experiments were performed in compliance with UK Home Office guidelines by Morwenna Muir. Animals were sacrificed as determined by UK Home Office regulations. The mice were weighed and monitored twice weekly. On cull, tissue was removed, weighed and stored in formalin for embedding or flash frozen in liquid nitrogen and stored at -80°C for further analysis. Fixed tissues were processed by the histology department at the Institute of Genetics and Molecular Medicine. Hematoxylin and eosin (H&E) staining was also carried out by the histology department.

2.2.1 Experimental metastasis assay

Female NOD SCID mice were inoculated intravenously via the tail vein with $0.5x10^6$ MDA-MB-231 shRNA Kin-1 (2 groups, *n*=4). The diet of one of the groups receiving MDA-MB-231 shRNA K1A cells lines was changed from a control diet (RM1 diet made by blending wheat, barley, wheatfeed, de-hulled extracted toasted soya, soya protein concentrate, macro and micro minerals, soya oil, whey powder, amino acids, and vitamins) to a doxycycline containing diet (RM1 + 625ppm Doxy (P) 25kGy) after 24 hours. The mice were culled 2 weeks post-implantation (carried out by M. Muir). QuPath v.0.2.0 was used to analyse the area of micrometastases in the lungs of mice.

2.2.2 Subcutaneous implantation

2.2.2.1 MDA-MB-231 cells

Ten female CD-1 Nude mice were inoculated subcutaneous bilaterally with $4x10^6$ MDA-MB-231 shRNA Kin1 cells. Group A (n=5) received the control RM1 diet and group B (n=5) received doxycycline containing diet (RM1 + 625ppm Doxy (P) 25kGy) from day 0. The mice were culled at day 25 (carried out by M. Muir).

Female NOD SCID mice were implanted subcutaneous bilaterally with MDA-MB-231 shRNA Kin1 fragments. Group A (n=5) received the control RM1 diet, group B (n=5) received the doxycycline containing diet (RM1 + 625ppm Doxy (P) 25kGy) from day 0 and group C (n=5) received the doxycycline containing diet (RM1 + 625ppm Doxy (P) 25kGy) from day 7. The mice were culled at the maximum end point (carried out by M. Muir).

2.2.2.2 Met-1 Clone A1 cells

Female FVB/NHanHSD mice were implanted subcutaneous bilaterally with $1x10^6$ Met-1 cells per implant. Group A (n=8) were inoculated with Met-1 Kin1-WT cells, group B (n=8) with Met-1 Kin1-Null cells and group C (n=8) with Kin1-AA cells. The mice from each group were culled for tissue on day 21.

Female CD-1 Nude mice were implanted subcutaneous bilaterally with $0.5x10^6$ Met-1 cells per implant. Group A (n=6) were inoculated with Met-1 Kin1-WT cells, group B (n=7) with Met-1 Kin1-Null cells and group C (n=7) with Kin1-AA cells. The mice from each group were culled for tissue at the maximum end point.

2.2.2.3 Met-1 Clone 3B cells

FVB/NHanHSD female mice were implanted subcutaneous bilaterally with $1x10^6$ Met-1 cells per implant. Group A (n=8) were inoculated with Met-1 Kin1-WT cells, group B (n=8) with Met-1 Kin1-Null cells and group C (n=8) with Kin1-NLS cells. Five mice from each group were culled for tissue on day 10, the remaining 3 mice continued for the growth curve.

2.3 Western Blotting

Cells were washed twice with cold PBS and lysed in RIPA buffer (50 mM Tris-HCl pH 8.0 (Sigma-Aldrich), 150 mM NaCl (Sigma-Aldrich), 1 mM EDTA (Sigma-Aldrich), 0.5%

sodium deoxycholate (Thermo Fisher Scientific), 0.1% SDS (Sigma-Aldrich) supplemented with 1.25 mM PMSF (complete ULTRA tablet, Roche), 1.7 µg/mL aprotinin (Sigma-Aldrich), 0.5 mM NaF (Sigma-Aldrich) and 0.1 mM sodium orthovanadate (Sigma-Aldrich). The lysed cells were centrifuged at 13,000 rpm for 15 minutes at 4 °C and the pellet discarded. Cell lysate protein concentration was determined by a bicinchoninic acid (BCA) assay (Invitrogen) and 20µg protein was added to 1x sample buffer (10 mM Tris-HCl pH6.8 (Sigma-Aldrich), 20% glycerol (ThermoFisher Scientific), 8% SDS (Sigma-Aldrich), 0.008% bromophenol blue (BioRad), 6% β-mercaptoethanol (Sigma-Aldrich)) and made up to 13µL with RIPA buffer. The samples were boiled at 65°C for 5 minutes and lysates resolved by gel electrophoresis. The gels were transferred to nitrocellulose (BioRad) and probed with primary antibodies in 5% BSA (Table 8) and membranes were then probed with anti-rabbit (1:5000; Cell Signaling Technology; 7074) or anti-mouse (1:5000; Cell Signaling Technology; 7076) for detection by chemiluminescence using a Bio-Rad ChemiDoc system. Blots were washed 3x 15 minutes with 1x Tris-buffered saline containing 0.1% Tween 20 (Sigma) detergent (T-BST) before imaging. A colorimetric image was a taken after the chemiluminescence image and the images merged to determine the molecular weight of the bands. Fiji was used to quantify protein levels and statistical analysis performed using GraphPad Prism v.8.0.2.

2.4 Real-Time Quantitative Reverse Transcription PCR

Reverse transcription and quantitative real-time PCR Total RNA isolation (Invitrogen) and cDNA synthesis (Superscript III RT-PCR kit, Invitrogen) were performed according to manufacturer's protocols. A NanoDrop2000 spectrophotometer was used to measure the concentration of RNA and cDNA. cDNA was mixed with 2X Mastermix (Applied Biosystems) and primers for the target gene or endogenous control (Table 9). Quantitative real-time PCR was performed in triplicate using an Applied Biosystems StepOne System (Applied Biosystems, Carlsbad, CA USA). SybrGreen reagent (Applied Biosystems) was used to detect gene expression and values were

normalised to the housekeeping gene, *Gapdh/GAPDH*. The cycling parameters used were as follows: 95°C for 5 seconds, 60°C for 10 seconds then 72°C for 10 seconds, for 40 cycles. The relative DNA expression was calculated using the Applied Biosystems StepOne software. The experiment was carried out on three independent occasions for each primer set unless otherwise stated. Analysis was performed using excel and GraphPad Prism v.8.0.2.

Table 8. Antibodies and dilutions used for western blotting.

Protein	Supplier	Catalogue Number	Dilution
Kindlin-1	Abcam	ab68041	1:1000
β-actin	Cell Signalling Technology	3700	1:5000
FAK	Cell Signalling Technology	3285	1:1000
ILK	Cell Signalling Technology	611802	1:2000
Kindlin-1	Movarian Biotech	-	1:500
GM130	BD Transduction Laboratories	610822	1:1000
α-tubulin	Cell Signalling Technology	2144	1:1000
Histone H4	Cell Signalling Technology	2592	1:1000
Kindlin-2	EMD Millipore	MAB2617	1:1000
p65	Cell Signalling Technology	8242	1:1000

2.5 Immunostaining

2.5.1 Immunohistochemistry

Paraffin sections were dewaxed with 2 x 5 minute washes of xylene (Fischer Scientific), and then rehydrated with 2 minute washes in decreasing concentrations of ethanol (100%, 100%, 80% and 50%). Sections were rinsed in water and then boiled for 5 minutes using a pressure cooker in pre-warmed 10 mM sodium citrate buffer (pH 6.0) (Fisher Scientific). Sections were cooled and washed in tap water followed by 2 x 5 minute washes in 0.0025% Tween in TBS (TBS-T). A hydrophobic pen (DAKO) was used to draw around sections before treating with peroxidase block (DAKO) for

15 minutes at room temperature. Sections were subsequently washed with water and incubated with serum free protein block (DAKO) for 2 hours at room temperature. Sections were incubated overnight at 4 °C with primary antibodies (Table 10) diluted in antibody diluent buffer (DAKO). A negative control was included in all experiments, in which a section was incubated with serum free protein block (DAKO) overnight. Following overnight incubation, sections were washed twice in 0.0025% TBS-T and incubated with either a mouse or rabbit Envision (DAKO) secondary antibody for 1 hour at room temperature. Sections were then washed 3 x 5 minutes with TBS-T and treated with DAB-DAB chromagen (DAKO) for 10 minutes at room temperature before counterstaining with haematoxylin (Pioneer Research Chemicals) for 2 minutes. Sections were rinsed with water and incubated for 2 minutes with Scott's tap water (0.35% sodium bicarbonate (Sigma-Aldrich) and 2% magnesium sulphate (Sigma-Aldrich) in distilled water). Sections were washed with water and dehydrated with 2 minutes washes in a series of solutions of increasing ethanol concentrations (50%, 80%, 100% and 100%). Finally, sections were washed twice for 2 minutes in xylene before being mounted in DPX mounting medium (Fisher). Sections were scanned using NanoZoomer-XR Digital slide scanner (Hamamatsu) and visualised using NDP.view 2 software U12388-01 (Hamamatsu).

2.5.2 Immunofluorescence

Cells were seeded onto glass coverslips in 6 well plates 24 hours in advance. Cells were fixed in PEM (80mM PIPES pH6.8, 5mM EGTA, 2mM $MgCl_2$) with 4% formaldehyde for 37°C for 15 minutes and then washed twice with PEM. Cells were then incubated with 2mL 0.1M glycine in PEM for 5 minutes at room temperature. Cells were permeabilised with 0.2% Triton X-100 in PEM for 5 minutes at room temperature. Cells were washed twice with PEM and then blocked with 2% BSA in PEM for 1 hour at room temperature. Coverslips were then incubated with primary antibodies (FAK Cell Signalling Technology; 3285 or ILK Santa-Cruz; sc-137221) overnight at 4°C on parafilm in a humidified container. Coverslips were washed three

times with 0.05% Tween-20 in PEM for 5 minutes at room temperature. The coverslips were incubated with 100µL of 1:400 secondary antibody (Alexa Fluor; 488 goat anti-rabbit IgG (H&L); Invitrogen; A11008 or Alexa Fluor; 488 goat anti-mouse IgG (H&L); Invitrogen; A11005) in 0.05% Tween 20 in PEM for 45 minutes at room temperature in the dark. Coverslips were then washed three times with 0.05% Tween 20 in PEM for 5 minutes at room temperature in the dark. The coverslips were washed with PEM and then fixed in PEM with 4% formaldehyde for 15 minutes. The coverslips were washed twice with PEM and incubated in 2mL 0.1M glycine in PEM for 5 minutes. The coverslips were washed with PEM and then water before mounting with fluoroshield containing DAPI (Sigma-Aldrich; F6057-20ML). Images of cells were taken using an Olympus FV3000 using a 60x 1.42 NA oil immersion objective with appropriate excitation and filter sets used.

2.6 Mammosphere Assay

Mammospheres were grown by plating 5000 cells per well on ultra-low-adhesive plates (Costar) as described by Shaw *et al.* (212) (Fig. 5A). Mammospheres were cultured in phenol red free DMEM/F12 medium (Invitrogen) supplemented with 5µg/mL insulin (Invitrogen), 20ng/mL epidermal growth factor (EGF) (Sigma-Aldrich), 10ng/mL basic fibroblast growth factor (FGF) (Preprotech) and 2% vol/vol B27 (Invitrogen). Mammospheres were cultivated for 5 days and imaged using an ImageExpress Micro (Molecular Devices) before passaging and reseeding to quantify their self-renewal. Secondary mammospheres were reseeded at the same density as the primary mammospheres and cultivated for 5 days. For experiments using the gamma secretase inhibitor, dibenzazepine (DBZ), 10µM of DBZ inhibitor (73092; Stem Cell Technologies) or an equal volume of Dimethyl sulfoxide (DMSO) was added to the media of the plates. Spheroids were counted using CellProfiler 3.0.0 (213) and calculations performed for analysis as described by Shaw *et al.* (212). GraphPad Prism v.8.0.2 was used for statistical analysis.

Table 9. Primers used for Real-Time Quantitative Reverse Transcription PCR

Target gene name	Primer description	Sequence (5'-3')
Mouse		
Fermt1	Forward	TGGAGAGCAGCAGCAGACAGAGA
	Reverse	GAAGAGAAGGTTGGCGTCAGCTG
Gapdh	Forward	ATGGTGAAGGTCGGTGTGA
	Reverse	AATCTCCACTTTGCCACTGC
Gata3	Forward	GCTCTTGCTACTCAGGTGAT
	Reverse	GGAGGGAGAGAGGAATCCGA
Msi1	Forward	AGATGTTCATCGGGGGACT
	Reverse	CCGAACTGGCCGAAGTATT
Lgr5	Forward	TCCTGTCCATTTTTGCTTCC
	Reverse	TGACAGAAAAACCTCGTTCCA
Wnt5a	Forward	ACGAGGAGCCATGTTCAGAA
	Reverse	ACGCAGGAGGATAACAACCA
Tnc	Forward	CCATGAAGGGATTCGAAGAA
	Reverse	TCATGCAGCTCGTACTCCAC
Nanog	Forward	GGGAAAAAGCCAGAAGTCG
	Reverse	CTTTGGGGACAAGCTGGA
Dtx1	Forward	CAGTGTCCAACCTGCAAAGC
	Reverse	CACCTTTCGGCCCTTCTCAT
Il-6	Forward	CCAGTTGCCTTCTTGGGACT
	Reverse	GAATTGCCATTGCACAACTCT
Human		
FERMT1	Forward	TGACCCAGACCAGCCTAAAG
	Reverse	ACTCTGGGTTGGGATTCAGG
GAPDH	Forward	GAGTCAACGGATTTGGTCGT
	Reverse	TTGATTTTGGAGGGATCTCG
IL-6	Forward	AGAGGCACTGGCAGAAAACA
	Reverse	TCACCAGGCAAGTCTCCTCA
TNC	Forward	GCTCAAAGCAGCCACTCATT
	Reverse	CCCATATCTGGAACCTCCTCT
Other		
RFP	Forward	AGCTGGACATCACCTCCCACAACG
	Reverse	GTACTGGAACTGGGGGGACAG

Table 10. Antibodies and concentrations used for immunohistochemistry

Protein	Supplier	Catalogue Number	Dilution
Kindlin-1	Abcam	ab68041	1:200
B220	Biolegend	103202	1:500
CD3	Abcam	ab16669	1:100
Kindlin-1	Movarian Biotech	N/A	1:100
Tenascin C	Abcam	ab108930	1:2000
RFP	Abcam	ab62341	1:400
Lamin A+C	Abcam	ab8984	1:500

2.7 Immunoprecipitation

2.7.1 Immunoprecipitation-mass spectrometry

Cells (Met-1 Kin1-Null, Met-1 Kin1-WT, Met-1 Kin1-AA, MDA-MB-231 4173 and MDA-MB-231 parental cells) were plated onto 15 cm dishes. Cells were cultured until they reached the desired confluency and lysed in 350µL RIPA buffer. 1mg of lysate was diluted in RIPA buffer with protease and phosphatase inhibitors and made up to a final volume of 500µL. Either 1µL Kindlin-1 (abcam; ab68041) or control rabbit IgG antibody (CST; 7074) was added to the lysate and samples were rotated at 4°C and then aliquoted into a deep-welled 96-well plate. Protein G Mag Sepharose™ Xtra beads (GE Healthcare) were washed with RIPA buffer (10µL per sample) and then resuspended in 100µL and aliquoted directly into the deep-welled 96-well plate. The samples were loaded into the Thermo Kingfisher Duo robot and samples with beads were washed 2x with RIPA buffer and 3x with TBS (50 mM tris, 150 mM NaCl, pH 7.4). Then the samples were digested in trypsin working reagent (2 M urea, 100 mM tris, 1 mM DTT, 6 ug/mL trypsin) at 27^{o}C for 30 minutes to release beads and the released peptides then digested with 1µg of porcine trypsin MS-grade (Promega) for 9 hours at 37 ^{o}C. Peptides were next alkylated by adding iodoacetamide to 50 mM (room temperature for 30 minutes). The peptides were acidified by addition of 10% TFA and

10ug of the resulting peptide solution was loaded onto an activated C18 STAGE tip, and washed with 100uL 0.1% TFA. Peptides were desalted and purified using STAGE tip protocol (214). Peptides were eluted in 80% Acetonitrile (v/v), 01% TFA (v/v) and lyophilized in a Concentrator Plus (Eppendorf). Peptides were resuspended in 7,5µl, 0.1% TFA and analysed by LC-MS/MS on a Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (Thermo Fisher). Protein identification and quantification was performed by label-free quantification using MaxQuant software, version 1.6.1.0 (215,216) with trypsin as protease and carbamidomethyl cysteine set as a fixed modification by A. Bolado Carrancio. The mouse or human proteome file used was downloaded from Uniprot in August 2017. Other parameters were set as default. Statistical analysis was performed using Perseus (217). The values were transformed into log2 and the protein hits filtered to eliminate those which were not detected in at least 3 out of 4 replicates. A t-test was performed to determine potential interactors and the list was filtered by a 2-fold enrichment unless stated. Perseus version 1.6.10.50, R version 3.5.0 (218), STRING (219) and Venny (220) were used to present the data.

2.7.2 Manual Immunoprecipitation

One mg of protein was used per sample and samples made up to 500 µL with RIPA buffer. Magnetic beads (10µL per 1 mg sample) were washed twice with 100µL of 0.2% TritonX in PBS using a bench top centrifuge. The beads were then resuspended in 200µL of PBS with 1 µL of Kindlin-1 antibody (Abcam; ab68041) or 1 µL of IgG control antibody (CST; 2729) and incubated for 30 minutes at room temperature on a roller. Beads were then washed twice with RIPA buffer before resuspending in 200µL RIPA per sample. The coated beads were added to the protein samples and incubated for 45 minutes at room temperature on a roller. The samples were washed three times with lysis buffer and twice with PBS. As much liquid was removed as possible and samples resuspended in 10µL PBS with 4µL 6x loading buffer and samples boiled at 65°C for western blot analysis.

2.9 Nuclear Fractionation

Cells were plated on 15 cm dishes and cultured until reaching the desired confluency. Media was removed with cells on ice and cells washed with ice cold PBS. Cells were lysed very gently using a cell scraper and 500 µL Buffer A (20 mM Tris pH 7.5, 1 mM MgCl2, 1 mM EGTA) plus inhibitors (1.25 mM PMSF (complete ULTRA tablet, Roche), 1.7 µg/mL aprotinin (Sigma-Aldrich), 0.5 mM NaF (Sigma-Aldrich) and 0.1 mM sodium orthovanadate (Sigma-Aldrich)) and detergent 0.05% NP-40 (Calbiochem). Samples were rotated at 4^{o}C for 5 minutes and centrifuged at 4^{o}C, 800 x g for 4 minutes. The supernatant was transferred into a new tube and NP-40 added to give a final concentration of 1%. Samples were vortexed vigorously, centrifuged at 4^{o}C, 14,000 rpm for 10 minutes and the supernatant collected as the cytoplasmic fraction. The pellet was washed twice with 200 µL of Buffer A with inhibitors and detergent and centrifuged at 4^{o}C, 800 x g, 3 minutes. The pellet was washed with 200 µL of Buffer A with inhibitors but no detergent and centrifuged at 4^{o}C, 800 x g, 3 minutes. The pellet was gently resuspended in two volumes of Buffer B (10 mM Tris-HCl pH 7.4, 2.5 mM MgCl2, 1.5 mM KCl, 0.2 M LiCl) with inhibitors (1.25 mM PMSF (complete ULTRA tablet, Roche), 1.7 µg/mL aprotinin (Sigma-Aldrich), 0.5 mM NaF (Sigma-Aldrich), 0.1% Triton X-100 and 0.1% sodium deoxycholate and rotated at 4^{o}C for 15 minutes and then centrifuged at 4^{o}C, 2,000 x g for 5 minutes. The supernatant was transferred to a fresh microfuge tube (keeping the pellet) and centrifuged at 14,000 rpm for 10 minutes and collected as the perinuclear fraction. The pellet was then washed twice with 200 µL Buffer B plus inhibitors and detergents and once with 200 µL Buffer B plus inhibitors but no detergents and centrifuged 4^{o}C 2000 x g, 3 minutes. The pellet was then resuspended in two volumes of RIPA buffer (50 mM Tris-HCl pH 8.0 (Sigma-Aldrich), 150 mM NaCl (Sigma-Aldrich), 1 mM EDTA (Sigma-Aldrich), 0.5% sodium deoxycholate (Thermo Fisher Scientific), 0.1% SDS (Sigma-Aldrich) supplemented with 1.25 mM PMSF (complete ULTRA tablet, Roche), 1.7 µg/mL aprotinin (Sigma-Aldrich), 0.5 mM NaF (Sigma-Aldrich) and 0.1 mM sodium orthovanadate (Sigma-Alrich) and sonicated at 4^{o}C on high power (5 cycles: 30 seconds on; 30 seconds off) using a water bath Diagenode sonicator. The sample was then centrifuged at 4^{o}C,

14,000 rpm for 10 minutes and the supernatant collected as the nuclear fraction. Samples were analysed by western blot using the antibodies in Table 8. The purity of each fraction was confirmed using controls as described in chapter 5.

2.9 Statistical Analysis

All p values below 0.05 were considered as statistically significant and statistical analysis was performed using GraphPad Prism software, version 8.0.2 (GraphPad Software Inc., La Jolla, CA, USA) except for the mass spectrometry data, which was analysed in Perseus version 1.6.10.50 (as stated above). Data is presented as mean ± standard error (SEM) and statistically analysed by using either a Student's t-test, a one-way ANOVA or two-way ANOVA. A Shapiro-Wilk test of normality was not performed on the data.

Chapter 3 The Role of Kindlin-1 in Breast Cancer

The mechanisms by which Kindlin-1 mediates metastasis to the lungs in breast cancer remain relatively unknown. Recent work from the Brunton group showed that Kindlin-1 regulates the secretion of a number of factors linked to metastatic spread including tenascin-C, a protein which promotes metastatic outgrowth of DTCs (185). Mechanistically, Tenascin-C regulates stem cell signalling by controlling expression of musashi homolog 1 (MSI1), a positive regulator of Notch signalling and LGR5, a WNT pathway target gene (205). Tenascin-C was shown to enhance Notch signalling and promote metastasis by inhibiting JAK2-STAT5 signalling in breast cancer cells (205). Inhibition of JAK2-STAT5 deters inhibition of the positive regulator of both Notch and Wnt pathways, MSI1, and thus, increases stem cell signalling activity (205).

The Kindlin proteins are highly expressed in the mouse and human embryo during early stage events (193) suggesting that their expression may correlate with stem-like characteristics of cells and several studies have supported this idea (156,165). For example, Kindlin-1 expression has been shown to increase expression of the stem cell marker LGR5 in colorectal cancer. In this study, Kindlin-1 promoted EMT (a pathway that has been linked with stem cell properties of cancer cells) (165). Kindlin-1 downregulated epithelial markers such as E-cadherin and ZO-1 through TGF-β-mediated regulation of Snail, Slug and Twist (165).

To date, the role of Kindlin-1 in stem cell regulation in breast cancer has not yet been addressed and therefore, we sought to define a role for Kindlin-1 in stem cell activity both *in vitro* and further define its role in breast cancer *in vivo.*

Specific aims of this chapter include:

1. Use of the *in vitro* mammosphere assay and gene expression analysis to investigate breast cancer stem cell activity *in vitro*.

2. Develop and characterise the loss of Kindlin-1 in human breast cancer cell models.

3. To establish whether Kindlin-1 regulates primary tumour growth and outgrowth of micrometastases through use of a *FERMT1* conditional knockdown cell model *in vivo.*

3.1 Regulation of mammosphere growth by Kindlin-1 in a mouse model

As Kindlin-1 regulates secretion of tenascin-C, which is known to be prominent in stem cell niches (221–223) and required for pulmonary micrometastatic outgrowth (205,207), we used a mouse model of Kindlin-1 loss to investigate effects on stem cell activity. The *Fermt1* gene was excised from the Met-1 cell line using the CRISPR-Cas9 method to generate Kindlin-1 Null cells (Kin1-Null) (185). The Met-1 cell line has a metastatic phenotype and was derived from the transgenic MMTV-PyMT mouse mammary gland carcinoma model of breast cancer (185). Wild-type (WT) Kindlin-1 was re-expressed into Kin1-Null cells to generate Kindlin-1 WT cells (Kin1-WT) (185). Loss of Kindlin-1 expression was not found to have an effect on the formation of primary mammospheres in the mouse Met-1 cell model (Fig. 13), which suggests that Kindlin-1 does not affect stem cell or early progenitor activity. However, loss of Kindlin-1 significantly decreased the generation of secondary mammospheres, which represents mammosphere self-renewal (Fig. 13C) and suggested that Kindlin-1 has a role in stem cell self-renewal.

3.2 Inhibition of Notch signalling does not alter mammosphere formation

To test the functional implications of this finding, we performed gene expression analysis in secondary mammospheres (Fig. 14). Loss of Kindlin-1 expression did not change expression of the mammary differentiation marker, *Gata3*, or components of the Wnt pathway; *Lgr5* and *Wnt5*, but significantly increased expression of *Nanog*, a factor that is essential for the maintenance of pluripotency in stem cells (224,225). Kindlin-1 loss significantly decreased expression of *Msi1* (Fig. 14), an inhibitor of a negative regulator of the Notch pathway, NUMB (Fig. 15A) (205). Kindlin-1 loss also resulted in significantly decreased expression of *Tnc* (Fig. 14), which is in line with what we previously reported in Met-1 cells grown in adherent 2D monolayers (185). As expression of *TNC* in CSCs has also been shown to enhance expression of *MSI1* (205), we investigated whether Kindlin-1 regulates self-renewal in this model through regulation of the Notch pathway (Fig. 15A). We used a γ-secretase inhibitor (GSI), DBZ

(226) to assess whether inhibition of Notch signalling in cells expressing Kindlin-1 would reduce mammosphere self-renewal to that of Kin1-Null cells. GSIs inhibit the Notch pathway by preventing proteolytic cleavage of the Notch receptor by γ-secretase (Fig. 15A). This prevents the release of the Notch intracellular domain, which upon cleavage, enters the cell nucleus to modify gene expression (Fig. 15A) (227). To confirm that DBZ was inhibiting the Notch pathway and select a concentration to use in the mammosphere assay, cells were treated with different concentrations of DBZ for 3 days alongside a DMSO control (Fig. 15B). Increasing the concentration of DBZ reduced cleaved Notch1 but did not impact Kindlin-1 expression in Met-1 Kin1- WT cells (Fig. 15B). Addition of 10 μM DBZ inhibitor significantly reduced the expression of the Notch pathway target gene, *Dtx1,* in Met-1 Kin1-WT cells but had no significant effect on *Dtx1* expression in Kin1-Null cells (Fig. 15C). Notch inhibition did not alter the size of the mammospheres formed or decrease mammosphere formation (Fig. 16A-B), a result which has been previously reported with the use of GSIs in human breast cancer models (228), and there was no significant effect on mammosphere self-renewal (Fig. 16B). Inhibition of Notch had no significant effect on expression of *Fermt1* and *Tnc* in both Met-1 Kin1-Null and Kin1-WT cells (Fig. 16C).

Notably, we did not find a significant difference in mammosphere self-renewal between DMSO control Met-1 Kin1-Null cells and Met-1 Kin1-WT cells in the mammosphere assay using the GSI (Fig. 16B) as previously found in Figure 13C for untreated cells. Following this finding, the mammosphere assay was repeated with addition of an untreated control as well as the GSI, DBZ, and a DMSO control to ensure that addition of DMSO was not affecting mammosphere formation (Fig. 16D). Repetition of the assay with each of these conditions did not result in a significant difference between Met-1 Kin1-Null and Kin-1-WT cells secondary mammospheres as previously observed (compare Fig. 13C with Fig. 16D).

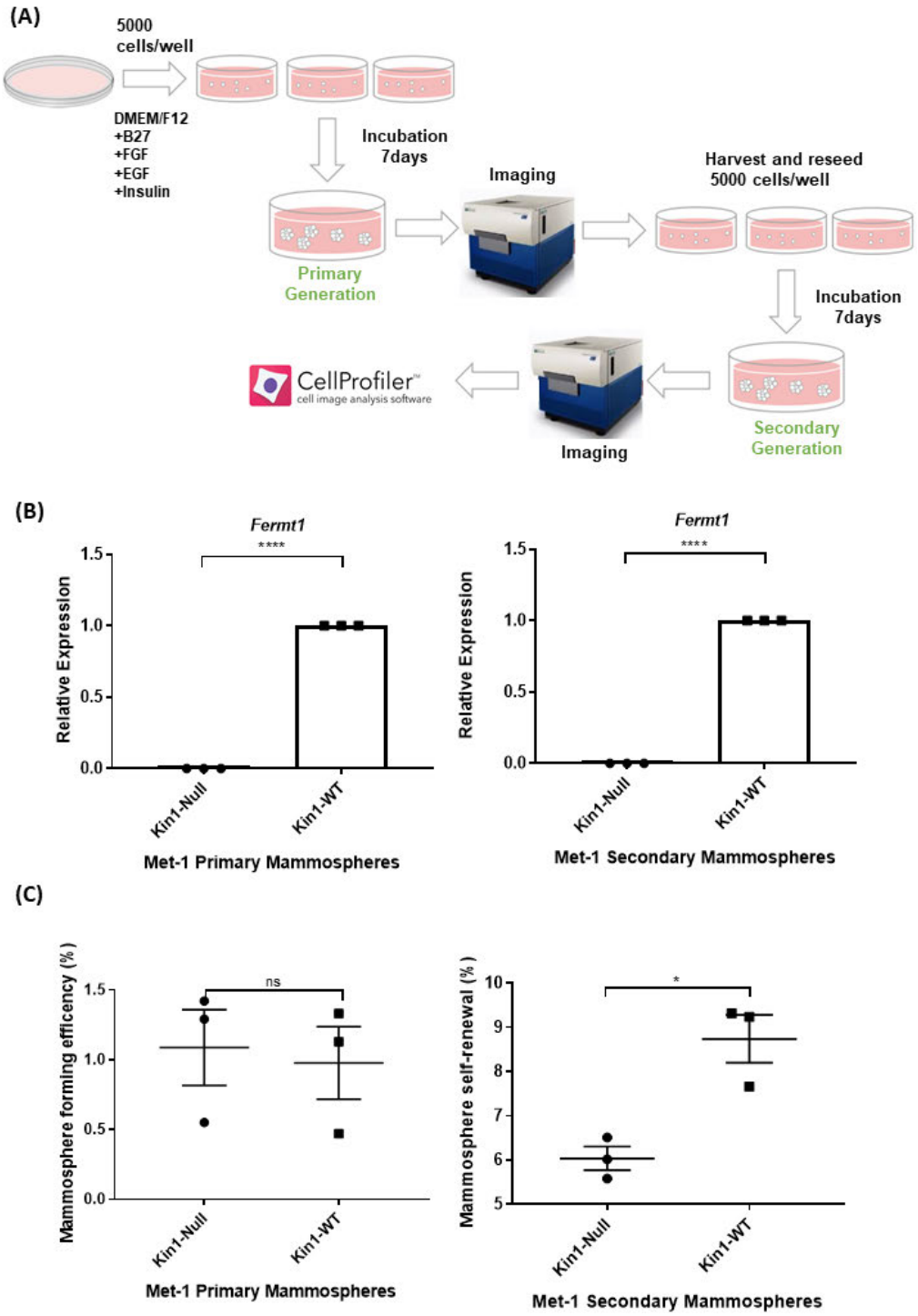


Figure 13. Regulation of mammosphere growth by Kindlin-1. (A) Schematic of the mammosphere assay (B) Kindlin-1 expression in mammospheres derived from Met-1 cells with genetic deletion of *Fermt1* (Kin1-Null) and re-expression of WT Kindlin-1 (Kin1-WT). Expression was analysed by qRT-PCR; values are mean ± SEM of triplicate experiments. The expression in Met-1 Kin1-Null cells was normalised to Met-1 Kin1-WT cells. (****p<0.0001). P values were obtained by a two-tailed Student's t test. (C) Quantitative analysis of mammosphere formation in primary and secondary spheroids in Kindlin-1 deficient Met-1 cells comparative to Met-1 Kin-WT. Combined data (average±SEM) from 3 independent experiments is shown for cell lines (*p<0.05). P values were obtained by two-tailed Student's t test.

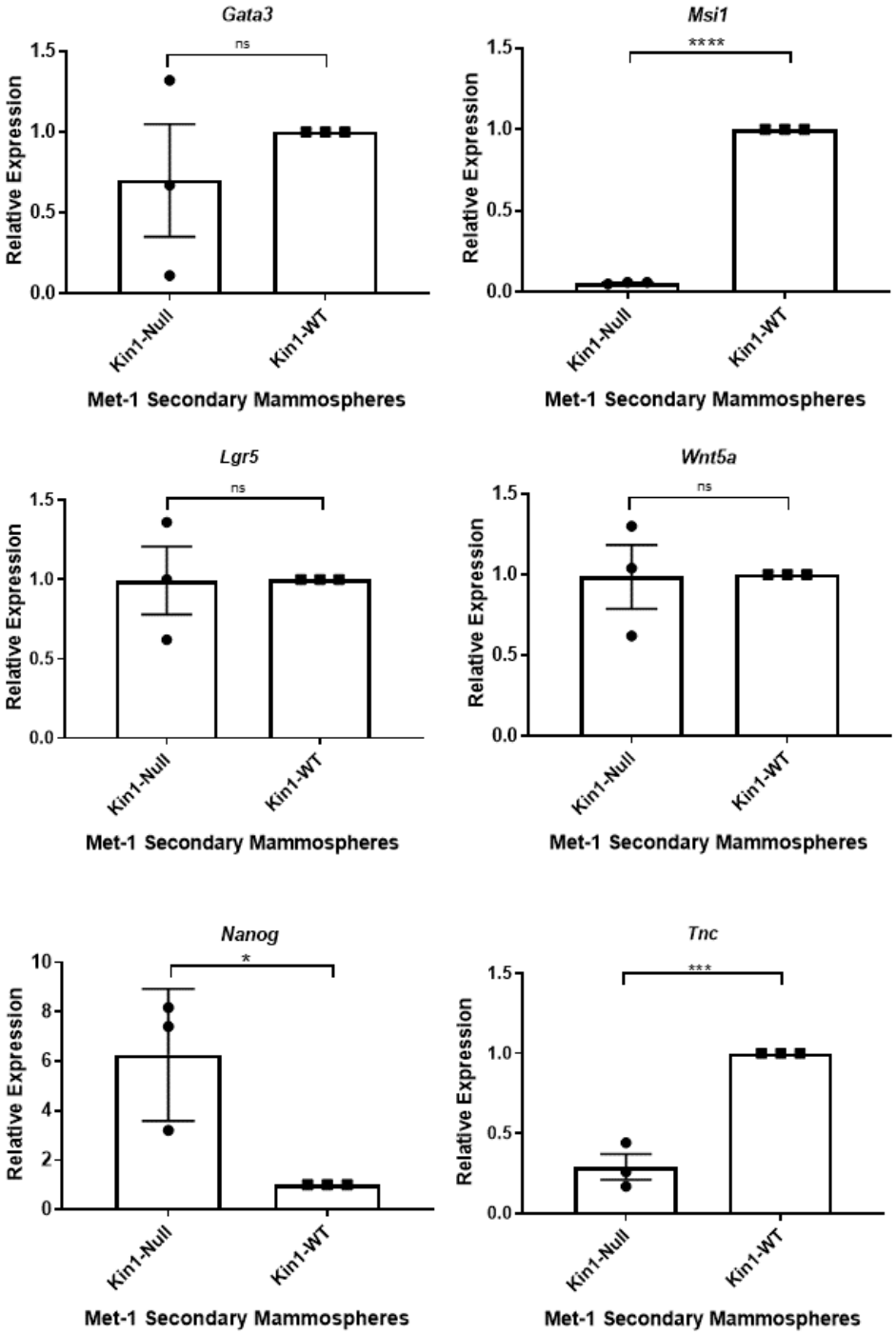


Figure 14. Gene expression analysis in Kindlin-1-deficient secondary mammospheres. Expression was analysed by qRT-PCR; values are mean ± SEM of triplicate experiments. For all genes, the expression in Met-1 Kin1-Null cells was normalised to Met-1 Kin1-WT cells. (**p*<0.05, ****p*<0.001, *****p*<0.0001). P values were obtained by a two-tailed Student's t test.

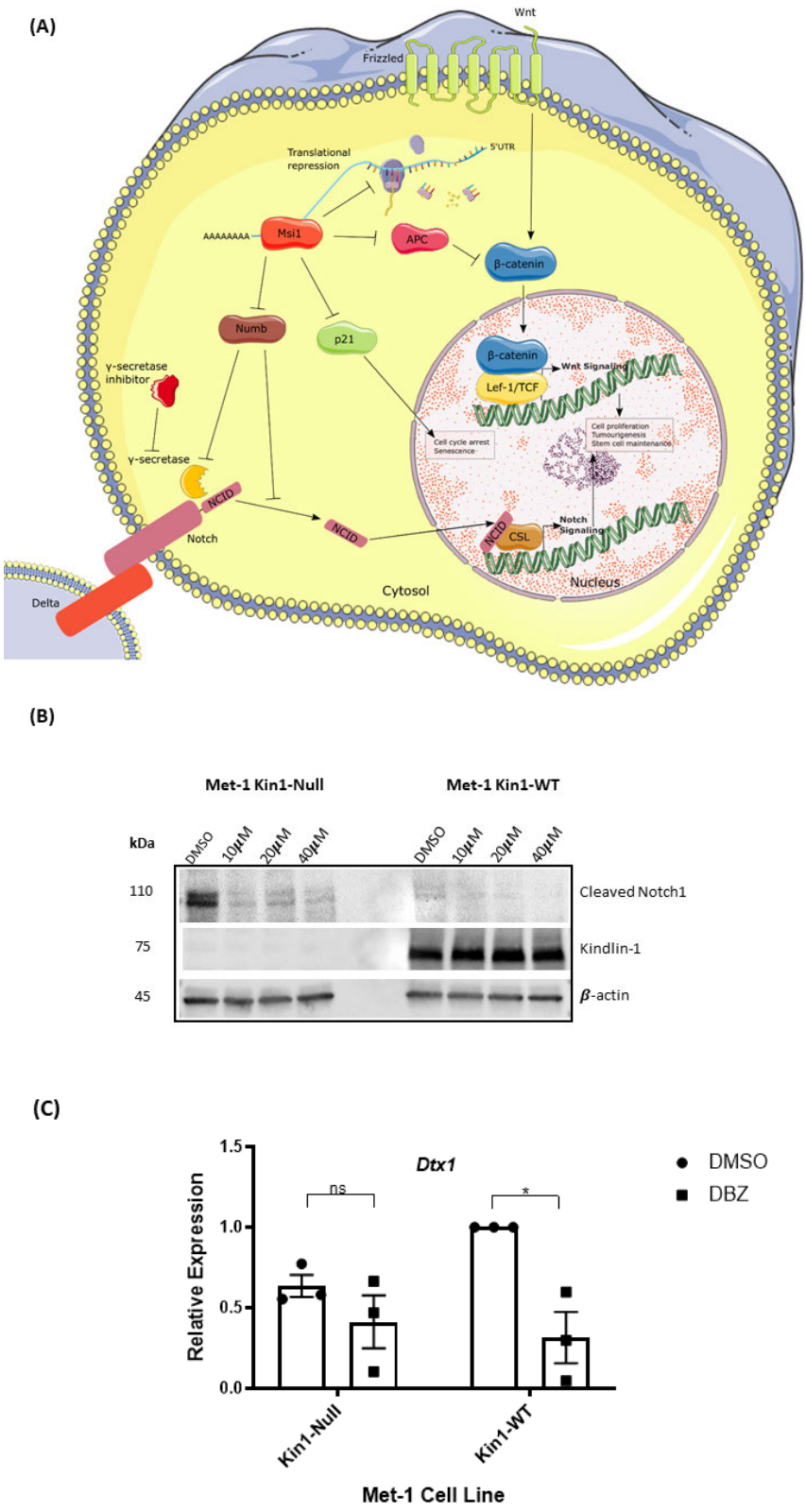
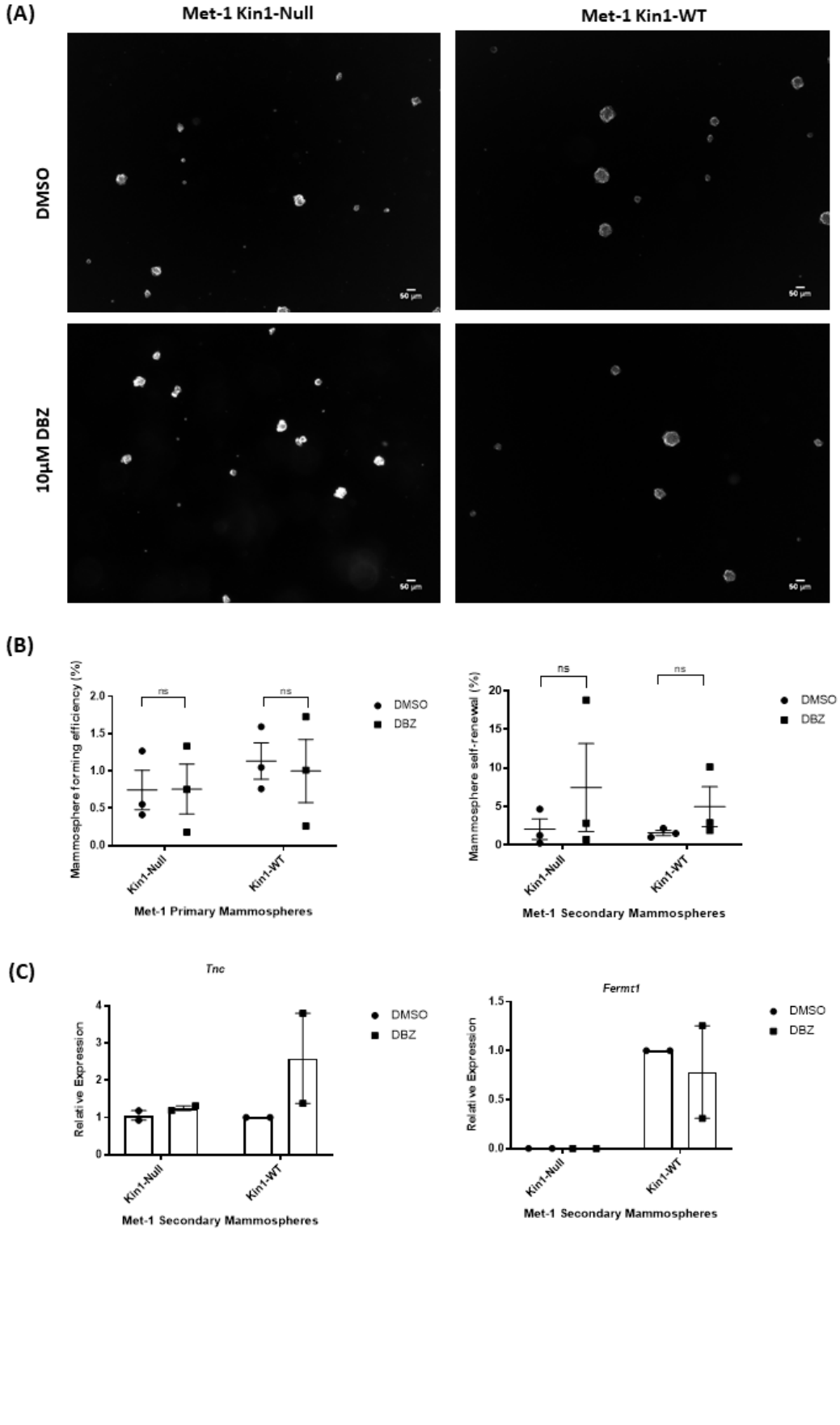


Figure 15. Inhibition of Notch signalling in Met-1 cells. (A) Working model of Musashi-1 (MSI1) in stem cell signalling. MSI1, is an RNA binding protein and stem cell regulator that positively regulates the Notch and Wnt signalling pathways by binding to the 3' untranslated region of target mRNAs and inhibiting their translation by preventing formation of the 80S ribosome complex. Target mRNAs

include *NUMB*, adenomatous polyposis coli (*APC*) and cyclin-dependent kinase inhibitor/p21WAF-1 (*CDKN1A*) and their downregulation promotes cell cycle progression through Wnt and Notch signalling pathways. The Notch pathway can be inhibited by targeting the enzyme complex, γ-secretase, with γ-secretase inhibitors. γ-secretase proteolytically cleaves the Notch receptor to release the intracellular domain, which subsequently translocates to the nucleus where it controls gene expression predominantly by binding to a ubiquitous transcription factor, CBF1, suppressor of hairless, Lag-1 (CSL). Transcription activators are recruited upon binding to the CSL complex and converts it from a transcriptional repressor into an activator, which turns on several downstream effectors. Figure created using Inkscape and Servier Medical Art. (B) Reduction of Notch signalling with addition of the γ-secretase inhibitor, dibenzazepine (DBZ). Western blot analysis for cleaved Notch1 expression and Kindlin-1 expression in Met-1 Kin1-Null and Kin1-WT cells after 3 days of treatment with DBZ or dimethyl sulfoxide (DMSO) as a control (left). β-actin was used as a loading control. (C) Expression of *Dtx1* after 3 days of treatment with 10 μM DBZ or DMSO was analysed by qRT-PCR; values are mean ± SEM of triplicate experiments. Gene expression in Met-1 Kin1-Null cells was normalised to Met-1 Kin1-WT cells. (**p*<0.05). P values obtained by a two-way ANOVA.


(A)
Met-1 Kin1-Null
Met-1 Kin1-WT
DMSO
10µM DBZ
50 µm
(B)
Mammosphere forming efficiency (%)
ns
DMSO
DBZ
Kin1-Null
Kin1-WT
Met-1 Primary Mammospheres
Mammosphere self-renewal (%)
Met-1 Secondary Mammospheres
(C)
Tnc
Relative Expression
Met-1 Secondary Mammospheres
Fermt1

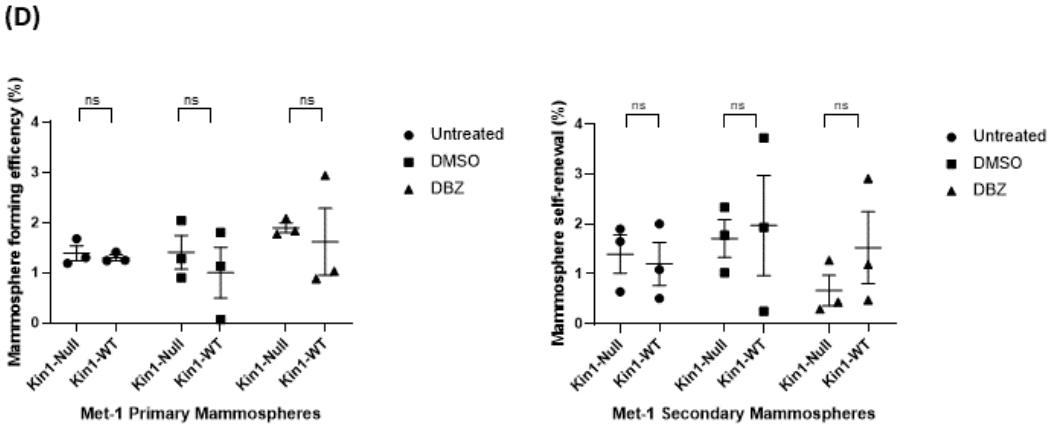
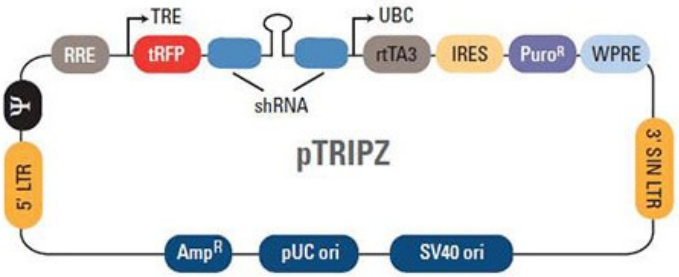


Figure 16. Inhibition of Notch signalling in Met-1 cells does not impact mammosphere formation. (A) Representative images illustrating the appearance of mammospheres treated with dimethyl sulfoxide (DMSO) or dibenzazepine (DBZ) after 3 days. Objective magnification: ×4. Scale bar represents 50µm. (B) Quantitative analysis of mammosphere formation in primary and secondary mammospheres in Kindlin-1 deficient Met-1 cells comparative to Met-1 Kin1-WT treated with 10 µM DBZ inhibitor or DMSO as a control for 7 days. Combined data (average ± SEM) from 3 independent experiments is shown for cell lines and a two-way ANOVA performed to test for statistical significance. (C) Gene expression analysis in Kindlin-1-deficient secondary mammospheres treated with DBZ inhibitor. Expression was analysed by qRT-PCR; values are mean ± SEM of two experiments. The expression in Met-1 Kin1-Null cells was normalised to Met-1 Kin1-WT cells. (D) Quantitative analysis of mammosphere formation in primary and secondary mammospheres in Kindlin-1 deficient Met-1 cells comparative to Met-1 Kin1-WT untreated, treated with 10µM DBZ inhibitor, or treated with DMSO as a control for 7 days. Combined data (average ± SEM) from 3 independent experiments is shown for cell lines and a two-way ANOVA performed to test for statistical significance.

3.3 Generation of an inducible human model of Kindlin-1 loss using shRNA

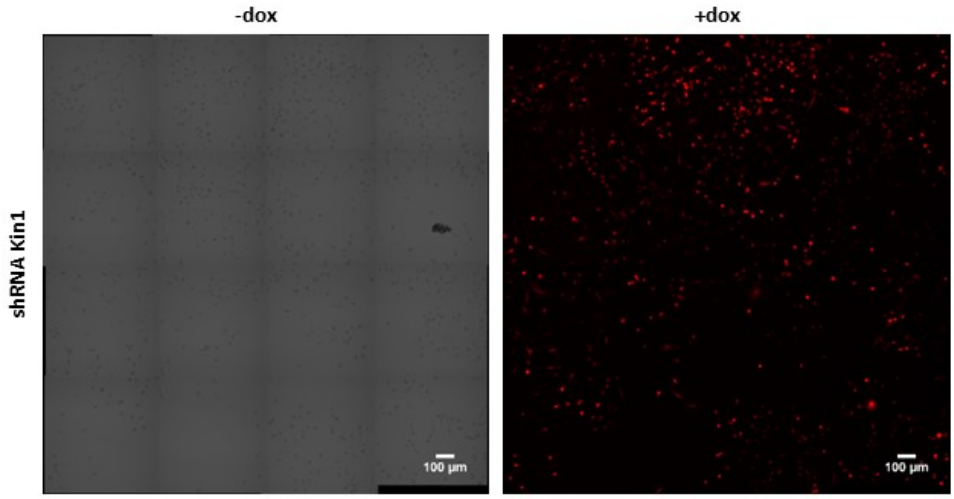
To understand further whether Kindlin-1 is involved in mammosphere formation, we generated another model of Kindlin-1 loss using human MDA-MB-231 cells with an inducible shRNA against Kindlin-1 (shRNA Kin1) (Fig. 17). The shRNA against Kindlin-1 is induced in the presence of doxycycline (Fig. 17C-D) and this also induces expression of red fluorescent protein (RFP) (Fig. 17A-B,D). A non-targeting shRNA (shRNA NT) was used as a control and upon treatment with doxycycline, significant reduction of Kindlin-1 was confirmed at the protein level in shRNA Kin1 cells (Fig. 17C). Gene expression analysis also confirmed significant reduction of *FERMT1* expression and increased expression in *RFP* in shRNA Kin1 cells (Fig. 17D).

(A)



Vector Element	Utility
TRE	Tetracycline-inducible promoter
tRFP	TurboRFP reporter for visual tracking of transduction and shRNA expression
shRNA	microRNA-adapted shRNA (based on miR-30) for gene knockdown
UBC	Human ubiquitin C promoter for constitutive expression of rtTA3 and puromycin resistance genes
rtTA3	Reverse tetracycline-transactivator 3 for tetracycline-dependent induction of the TRE promoter
Puro^R	Puromycin resistance permits antibiotic-selective pressure and propagation of stable integrants
IRES	Internal ribosomal entry site allows expression of rtTA3 and puromycin resistance genes in a single transcript
5' LTR	5' long terminal repeat
3' SIN LTR	3' self-inactivating long terminal repeat for increased lentivirus safety
Ψ	Psi packaging sequence allows viral genome packaging using lentiviral packaging systems
RRE	Rev response element enhances titer by increasing packaging efficiency of full-length viral genomes
WPRE	Woodchuck hepatitis posttranscriptional regulatory element enhances transgene expression in the target cells

(B)



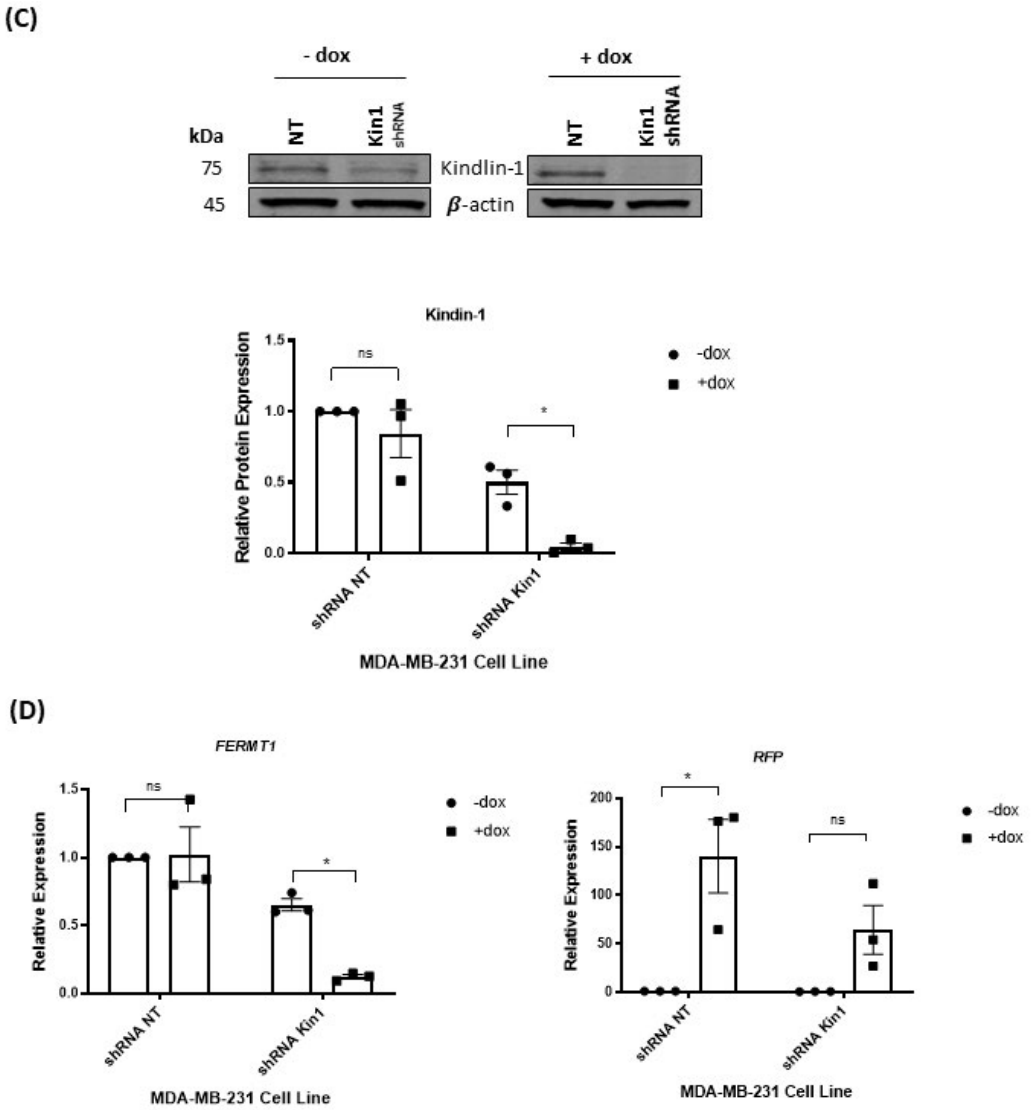


Figure 17. Knockdown of Kindlin-1 in MDA-MB-231 cells *in vitro*. (A) Vector map of TRIPZ Inducible Lentiviral shRNA. The vector is tetracycline (Tet)-inducible, which enables reversible control of gene silencing. shRNA expression is induced in the presence on doxycycline (Tet-On) alongside expression of turbo red fluorescent protein (tRFP). (B) Representative images of MDA-MB-231 shRNA Kin1 cells – dox and +dox after 24 hours. Cells express RFP upon addition of dox. Objective magnification: 10x. Scale bar represents 100µm. (C) Western blot analysis for Kindlin-1 expression in MDA-MB-231 shRNA Kin1 cells +/– doxycycline (dox) to induce knockdown of Kindlin-1 compared to a non-targeting shRNA (NT) control. ***β***-actin was used as a loading control. Kindlin-1 protein expression was significantly lower with addition of doxycycline in shRNA Kin1 cells. Expression was analysed by Fiji; values are mean ± SEM of three replicates. Protein expression was normalised to shRNA NT -dox cells. (**p*<0.05). P values were obtained by a two-way ANOVA. (D) Gene expression analysis of *FERMT1, RFP, TNC* and *IL-6* in MDA-MB-231 shRNA Kin1 cells –dox and +dox to induce knockdown of Kindlin-1 compared to a shRNA NT control for *FERMT1* and *RFP* expression. Expression was analysed by qRT-PCR; values are mean ± SEM of three experiments. Gene expression was normalised to NT -dox cells for *FERMT1* and *RFP* expression and Kin1 –dox cells for *TNC* and *IL-6* expression. (**p*<0.05). P values were obtained by a two-way ANOVA or a two-tailed Student's t test.

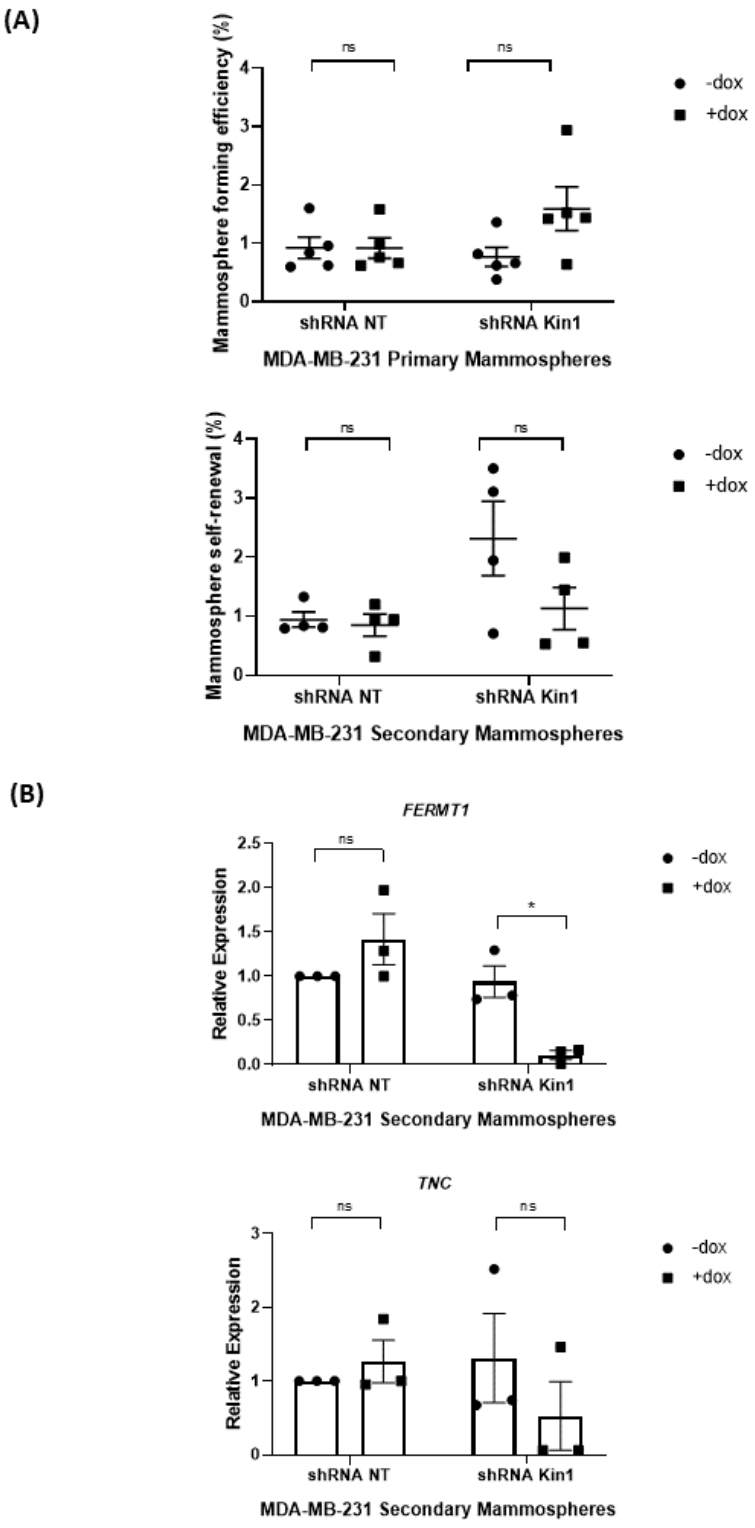


Figure 18. Kindlin-1 does not regulate mammosphere growth in MDA-MB-231 cells. (A) Quantitative analysis of mammosphere formation in primary and secondary mammopsheres in Kindlin-1 deficient MDA-MB-231 cells. Combined data (mean ±SEM) from 3 independent experiments is shown for cell lines and a one-way ANOVA performed. (B) Gene expression analysis in Kindlin-1-deficient secondary mammospheres. Expression was analysed by qRT-PCR; values are mean ± SEM of triplicate experiments. Gene expression was normalised to NT -dox cells and a one-way ANOVA performed.

3.4 Characterisation of the effect of Kindlin-1 on stem cell activity in MDA-MB-231 cells

We next used the inducible Kindlin-1 shRNA model to investigate Kindlin-1 loss on stem cell activity in MDA-MB-231 cells. Loss of Kindlin-1 did not have a significant effect on the formation of primary mammospheres and although there was a small reduction in secondary mammospheres with Kindlin-1 loss, this was not significant (Fig. 18). Furthermore, *TNC* expression was also unaltered by Kindlin-1 loss in secondary mammospheres (Fig. 18B).

3.5 Kindlin-1 and Tenascin C are highly expressed in human lung metastatic MDA-MB-231 cells

In order to explore further the role of Kindlin-1 in breast cancer, protein levels of Kindlin-1 in a lung metastatic variant of the human breast cancer cell line, MDA-MB-231, were compared to its parental counterpart (Fig. 19A). The lung metastatic variant was shown to have increased Kindlin-1 expression compared to its parental counterpart (Fig. 19A). Previously, the lung metastatic variant MDA-MB-231 cell line has been shown to have increased mammosphere secondary formation that has been linked to a greater *TNC* expression (205) and we were also able to confirm increased *TNC* expression (Fig. 19B). As we have previously shown that Kindlin-1 regulates *Tnc* in the Met-1 model, we hypothesised that Kindlin-1 may be important in the lung metastatic MDA-MB-231 variant, which is dependent on *TNC* for mammosphere formation. In order to investigate this hypothesis, CRISPR-cas9 technology was used to knock out Kindlin-1 in the lung metastatic variant of the human breast cancer cell line, MDA-MB-231 as it was shown to have increased Kindlin-1 expression compared to its parental counterpart (Fig. 19A,C). The pool of lung metastatic MDA-MB-231 CRISPR-Kindlin-1 cells generated, had a distinct decrease in expression of Kindlin-1 in comparison to the control cell lines generated (Fig. 19C). Single cell cloning of the pool of cells was attempted to identify clones with

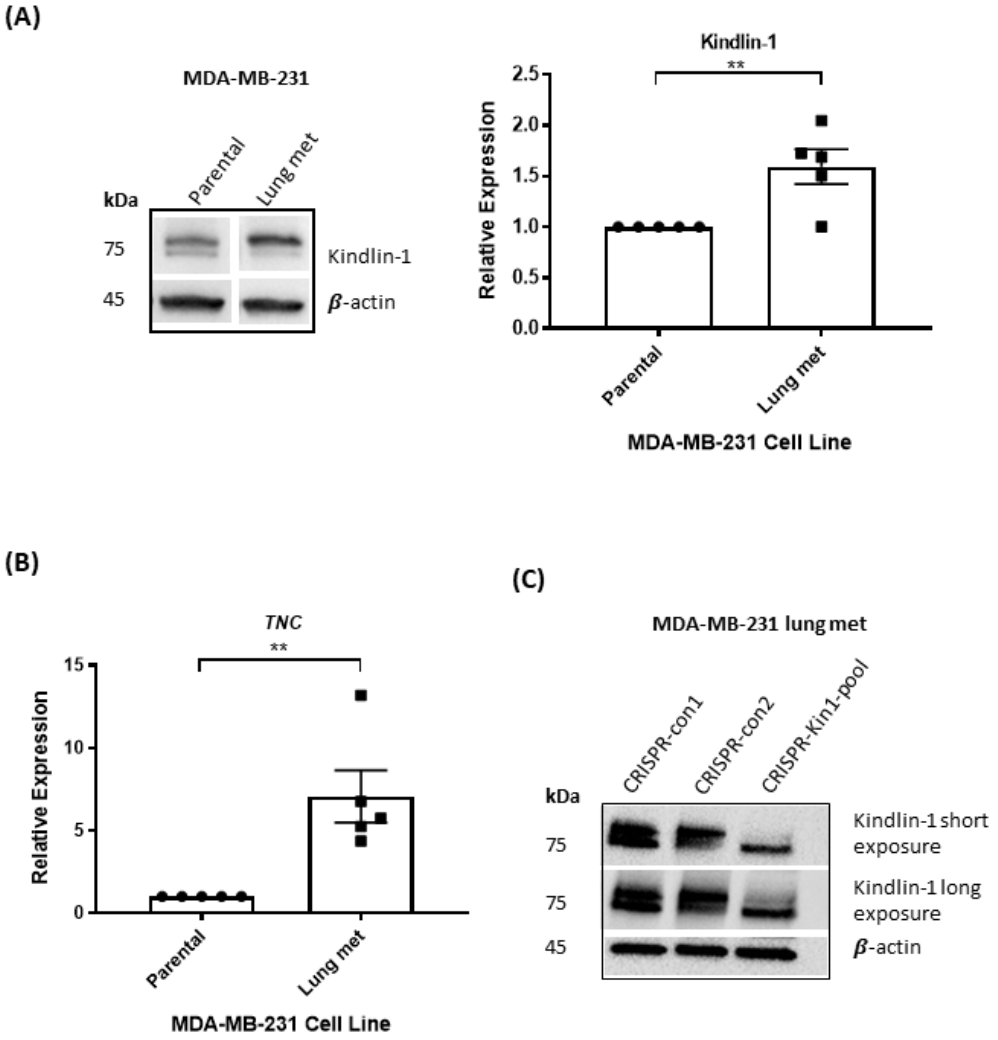


Figure 19. Kindlin-1 and tenascin-C expression is significantly higher in a human breast cancer cell line that has high capacity to colonise the lungs in mice. (A) Western blot analysis for Kindlin-1 expression in a lung metastatic variant of MDA-MB-231 cells compared to their parental counterpart (left). $\boldsymbol{\beta}$-actin was used as a loading control. Kindlin-1 protein expression quantification in the lung metastatic variant compared to their parental counterpart (right). Expression was analysed by Fiji; values are mean ± SEM of five replicates. Protein expression in lung metastatic cells was normalised to parental cells. (**p<0.01). P values were obtained by a two-tailed Student's t test. (B) Tenascin-C expression in the lung metastatic variant compared to their parental counterpart. Expression was analysed by qRT-PCR; values are mean ± SEM of five experiments. Gene expression in lung metastatic cells was normalised to parental cells. (**p<0.01). P values were obtained by a two-tailed Student's t test. (C) Western blot analysis demonstrating loss of Kindlin-1 expression in the lung metastatic variant of MDA-MB-231 cells. $\boldsymbol{\beta}$-actin was used as a loading control.

complete knockout of Kindlin-1, however, due to problems with the specificity of new batches of the Kindlin-1 antibody, confirmation of complete knockdown was difficult and the decision was made to generate a new Kindlin-1 antibody using Moravian

Biotech before proceeding. Time delays due to Covid-19 has meant that this has not been pursued further.

3.6 Characterisation of the effect of Kindlin-1 on the outgrowth of micrometastases

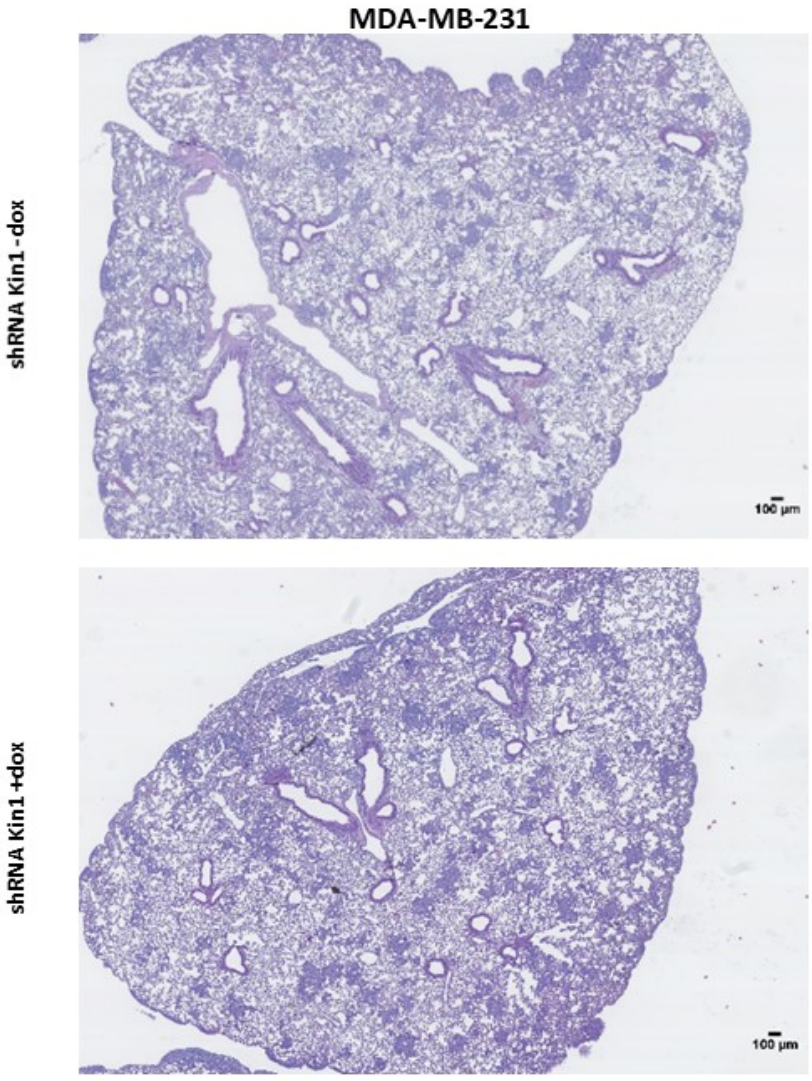
As the Brunton group previously demonstrated that pulmonary metastatic burden is reduced with loss of Kindlin-1 in the Met-1 model (185), we set out to establish whether Kindlin-1 regulates outgrowth of micrometastases in the MDA-MB-231 model. Mice were inoculated intravenously with MDA-MB-231 shRNA Kin1 tumour cells and the cells allowed to seed in the lungs for one day before knockdown of Kindlin-1 was induced. H&E were used to stain sections (Fig. 20A) and the area covered by metastases was confirmed by lamin A+C immunohistochemistry (Fig. 20C). Stained H&E sections were used to quantify the area covered by metastasis and after 14 days, there was no significant difference in percentage area of metastases found (Fig. 20B), however, IHC using a Kindlin-1 antibody showed that there was no reduction in Kindlin-1 expression (Fig. 20C). We also observed RFP staining in the metastases in the presence and absence of doxycycline suggesting that the promoter was leaky (Fig. 20C). Similar expression of tenascin C was observed in the two different groups (Fig. 20C).

In the previous experiment (Fig. 20C), IHC suggested that knockdown of Kindlin-1 was not achieved and we had concerns with regards to the specificity of the Kindlin-1 antibody and decided to confirm knockdown using RT-qPCR. The metastases formed in the lungs were too small to remove from the lungs to extract only RNA corresponding to the tumour cells and perform RT-qPCR for Kindlin-1 expression. Therefore, to assess whether sufficient knockdown of Kindlin-1 could be achieved *in vivo*, MDA-MB-231 shRNA Kin1 cells were injected into the mammary fat pad (Fig. 21). RNA was extracted from the tumour cells and RT-qPCR was performed for expression of both *FERMT1* and *RFP* using human specific primers (Fig. 21C). Kindlin-

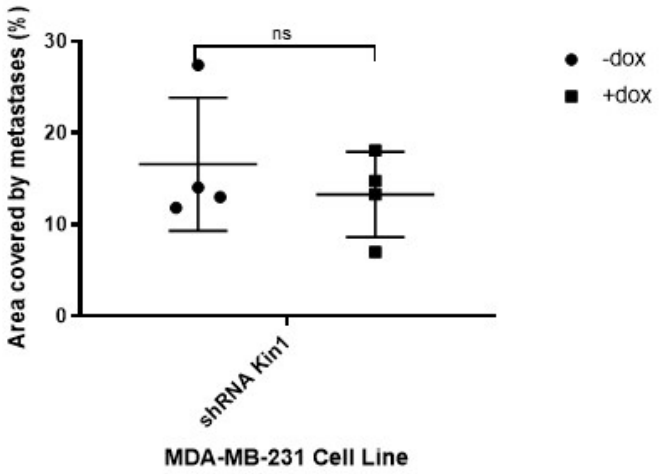
1 expression was significantly reduced and RFP expression significantly increased in mice receiving doxycycline compared with the control group (Fig. 21C).

Staining showed an increase in RFP expression with the addition of doxycycline, however, similar levels of Kindlin-1 expression were observed in both groups suggesting that the antibody was binding non-specifically (Fig. 22). The levels of tenascin-C expression in the mice receiving the doxycycline diet were similar to those receiving the control diet (Fig. 22). Tumours from the CD-1 nude mice receiving the doxycycline diet had a reduced growth rate compared to those receiving the normal diet suggesting that Kindlin-1 may regulate primary tumour growth in this model, however, only one time point in the experiment reached significance (Fig. 21A). Statistical analysis of the final tumour volume showed a significant reduction in volume at day 25 with loss of Kindlin-1 (Fig. 21B). The experiment was repeated to establish if the data was reproducible using SCID mice and MDA-MB-231 shRNA Kin1 tumour fragments were implanted into the mice to reduce the time taken for tumours to appear (Fig. 23). In this experiment, two of the groups received doxycycline to induce knockdown of Kindlin-1. One group received doxycycline at day zero to evaluate if knockdown of Kindlin-1 affects tumour initiation in this model and one group at day seven to evaluate if Kindln-1 knockdown affects the outgrowth of the tumour once it has been established (Fig. 23). There was no significant difference between the growth rates of either of these groups compared to mice receiving the control diet and the growth rates followed a similar trend (Fig. 23A). We also did not find a significant difference between the final tumour volumes of the groups (Fig. 23B). Analysis of mRNA confirmed that the knockdown had been successful as expression of Kindlin-1 was significantly reduced and RFP expression significantly increased in the groups receiving the doxycycline diet (Fig. 23C).

(A)


MDA-MB-231
shRNA Kin1 -dox
shRNA Kin1 +dox
100 µm
100 µm

(B)


Area covered by metastases (%)
30
20
10
0
ns
-dox
+dox
shRNA Kin1
MDA-MB-231 Cell Line

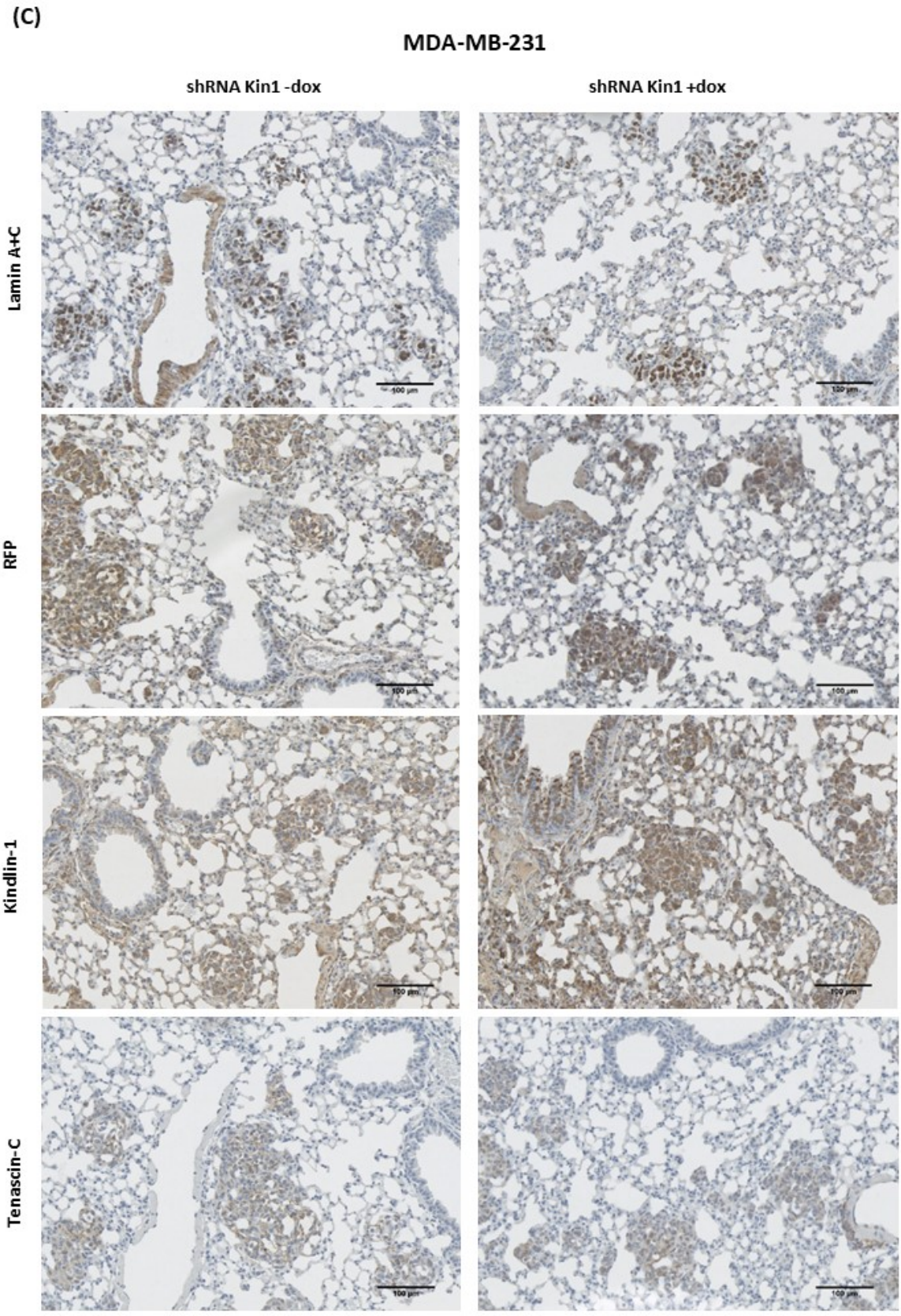


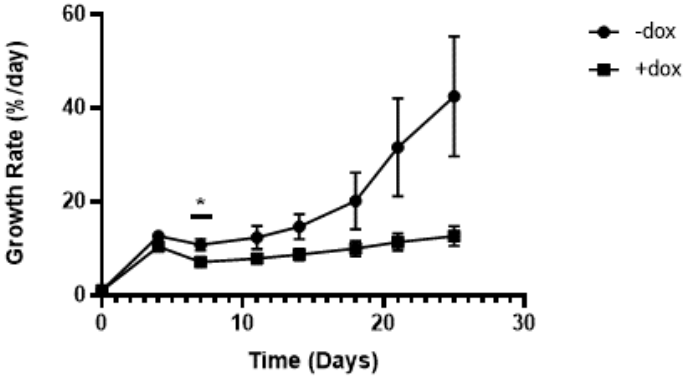
Figure 20. Pulmonary metastasis was not altered in mice inoculated with MDA-MB-231 shRNA Kin1 cells. (A) Representative haematoxylin and eosin staining of lung metastases from mice inoculated with MDA-MB-231 shRNA Kin1 cells –dox or +dox to induce knockdown of Kindlin-1. Objective

magnification: ×2.5. Scale bar represents 100µm. (B) Total area covered by metastatic lesions in lungs after injection of MDA-MB-231 shRNA Kin1 cells –dox and +dox. Values are means ± SEM (n=4 mice per group, *p*=ns, Student's t test). Quantification was performed using QuPath. (C) Immunohistochemistry staining of Lamin A+C, RFP, Kindlin-1 and Tenascin-C protein expression in lungs of MDA-MB-231 shRNA Kin1 cells –dox and +dox mice. Objective magnification: ×20. Scale bar represents 100µm.

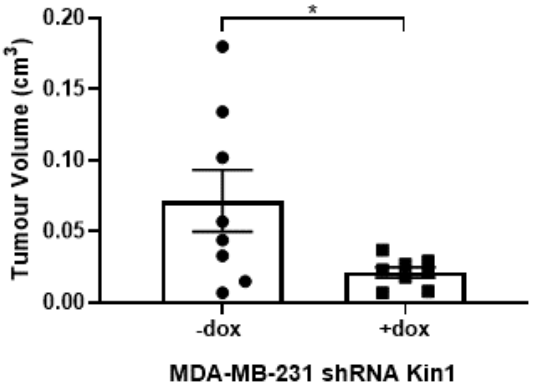
3.7 Tumour growth is significantly reduced with loss of Kindlin-1 in Met-1 cells in immunocompetent and immunodeficient mouse models and Kin1-Null tumours have significantly reduced regulatory T cells

We then proceeded to establish whether loss of Kindlin-1 affected primary tumour growth in a subcutaneous Met1 model. As described earlier in this chapter, a model of Kindlin-1 loss was previously generated in Met-1 cells and WT-Kindlin-1 re-expressed into Met-1 Kin1-Null cells to generate Met-1 Kin1-WT cells (185). Additionally, a mutant Kindlin-1, in which glutamine 611 and tryptophan 612 was mutated to alanine was also re-expressed into Met-1 Kin1-Null cells to generate Kindlin-1 AA cells (Kin1-AA) (185). Tryptophan 612 is critical for integrin binding and its mutation to alanine, renders it unable to bind β-integrin tails (145). The Kin-1 AA cell line enables the role of Kindlin-1 and integrin activation to be investigated in the cell line. Mice were inoculated with Met-1 cells subcutaneously and loss of Kindlin-1 reduced the growth rate in the immunocompetent FVB mice (Fig. 24A) and in the immunodeficient CD-1 nude mice (Fig. 24C). The final tumour volume was significantly reduced in both FVB (Fig. 16B) and CD-1 mice (Fig. 24D) that were inoculated with Met-1 Kin1-Null cells compared to mice inoculated with Kin1-WT and Kin1-AA cells. There was an initial time delay in the growth rate of tumours from mice inoculated with Met-1 Kin1-AA cells, which expressed a mutant Kindlin-1 that is unable to bind to integrin (Fig. 24A,C). The growth rate of Met-1 Kin1-AA tumours initially mirrored Kin1-Null tumours but Kin1-AA tumours later caught up to the growth rate of Kin1-WT cell tumours (Fig. 24A,C). This suggests that integrin activation by Kindlin-1 is required for tumour initiation but once the tumour has been established, integrin activation by Kindlin-1 is no longer required for tumour growth.

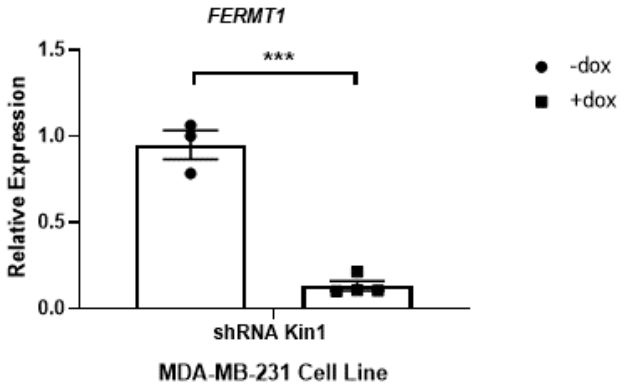
(A)



(B)



(C)



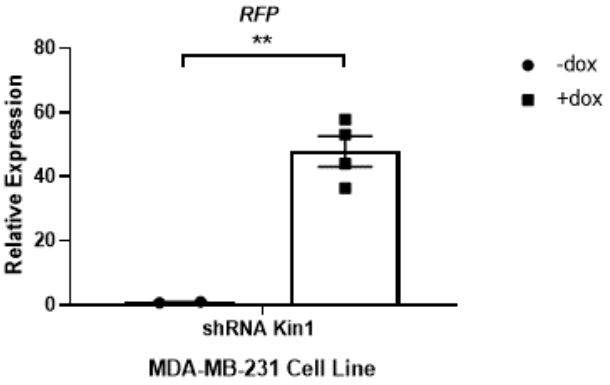


Figure 21. Kindlin-1 knockdown in CD-1 mice subcutaneously inoculated with MDA-MB-231 shRNA

Kin1 cells. (A) Mean (±SEM) tumour growth from mice inoculated with MDA-MB-231 shRNA Kin1 cells –dox or +dox to induce knockdown of Kindlin-1 (n=8 tumours per group, *$p<0.05$, two-way ANOVA). (B) Mean (±SEM) tumour volume at day 25 (n=8 tumours per group, *p<0.05, student's t test). (C) *FERMT1* and *RFP* expression in MDA-MB-231 shRNA Kin1 cells –dox or +dox tumours. Expression was analysed by qRT-PCR; values are mean ± SEM of 3 tumours for –dox cells and 4 tumours for +dox cells. Gene expression in lung metastatic cells was normalised to -dox cells. (**p<0.01). P values were obtained by a two-tailed Student's t test.

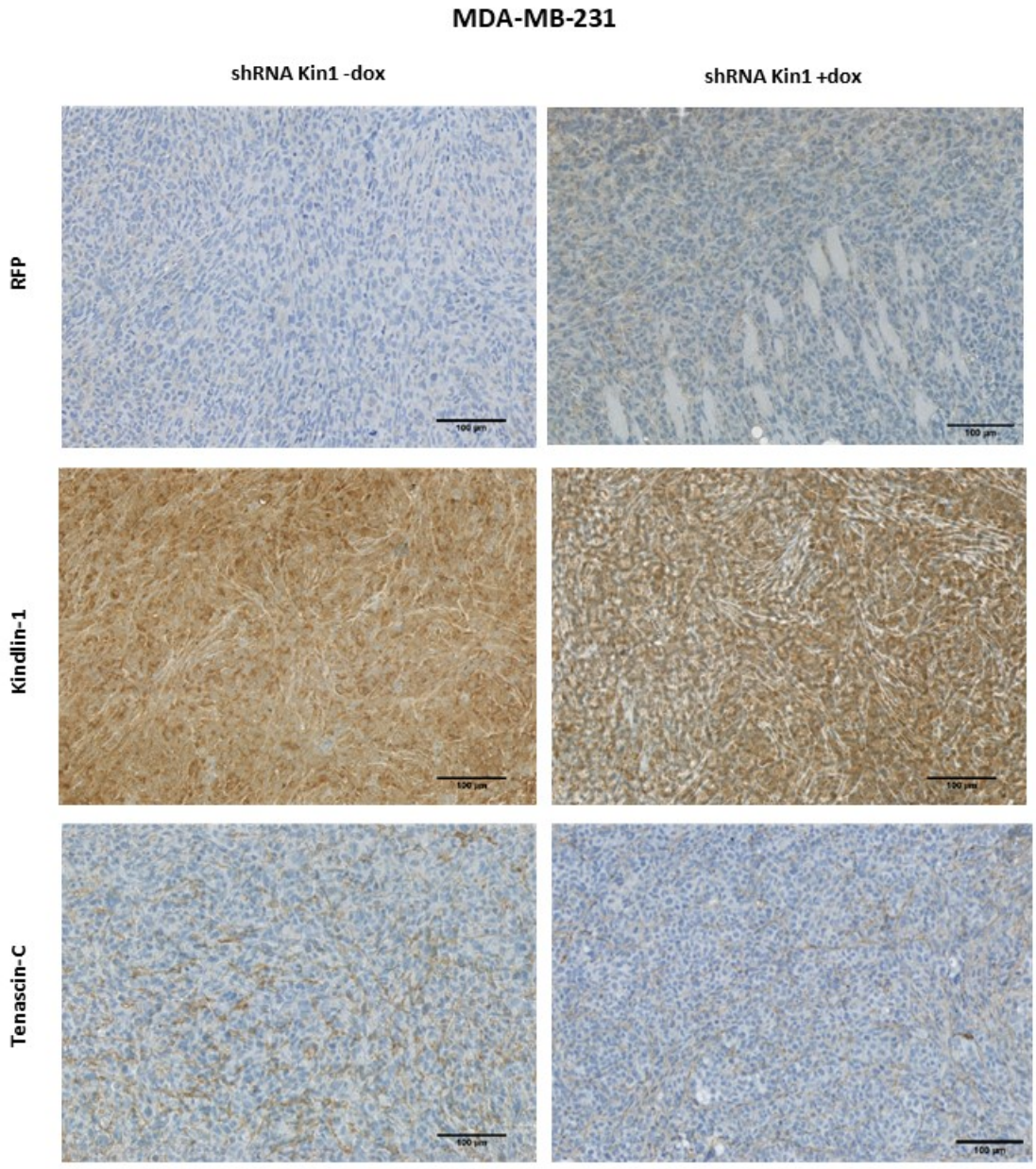
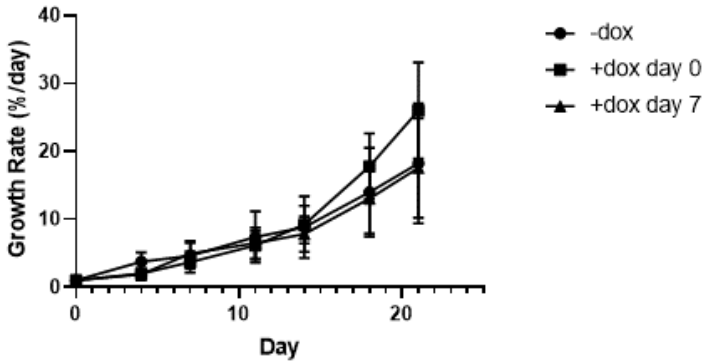
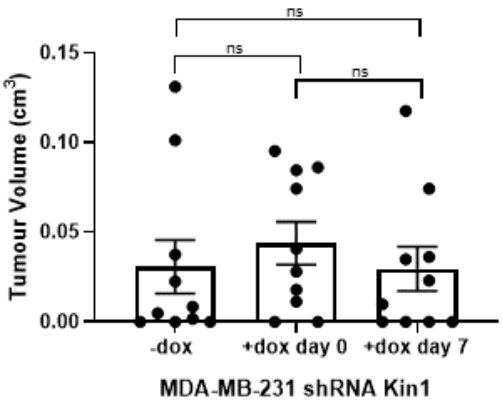


Figure 22. Immunohistochemistry staining of MDA-MB-231 shRNA Kin1 cells –dox and +dox subcutaneous tumours. Representative images of immunohistochemistry staining of RFP, Kindlin-1 and Tenascin-C in MDA-MB-231 shRNA Kin1 cells –dox and +dox subcutaneous tumours from CD1 nude mice. Objective magnification: ×20. Scale bar represents 100µm.

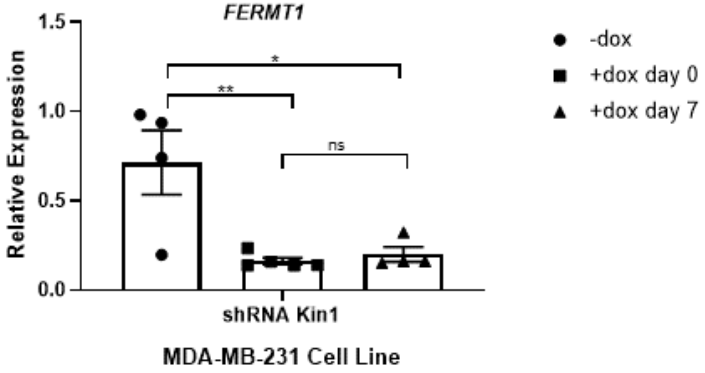
(A)


Growth Rate (%/day)
Day
-dox
+dox day 0
+dox day 7

(B)


Tumour Volume (cm3)
ns
ns
ns
-dox
+dox day 0
+dox day 7
MDA-MB-231 shRNA Kin1

(C)


FERMT1
Relative Expression
shRNA Kin1
MDA-MB-231 Cell Line
-dox
+dox day 0
+dox day 7

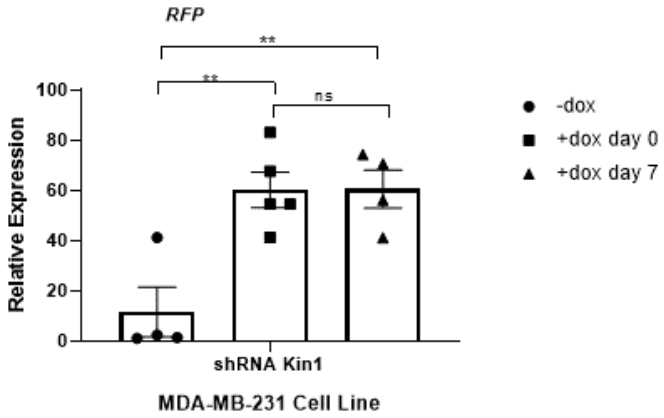

RFP
Relative Expression
shRNA Kin1
MDA-MB-231 Cell Line
-dox
+dox day 0
+dox day 7

Figure 23. Kindlin-1 knockdown in SCID mice subcutaneously inoculated with MDA-MB-231 shRNA Kin1 cells. (A) Mean (±SEM) tumour growth from mice inoculated with MDA-MB-231 shRNA Kin1 cells –dox or +dox at day 0 or day 7 to induce knockdown of Kindlin-1 (n=10 tumours per group, *p=ns,* two-way ANOVA). (B) Mean (±SEM) tumour volume at day 21 (n=10 tumours per group, one-way ANOVA). (C) Analysis of *FERMT1, RFP* and *IL-6* expression in MDA-MB-231 shRNA Kin1 cells –dox or +dox tumours. Expression was analysed by qRT-PCR; values are mean ±SEM of 4 tumours for –dox cells, 5 tumours for +dox day 0 cells and 4 tumours for +dox day 7 cells. Gene expression was normalised to -dox cells. (**p*<0.05,***p*<0.01, one-way ANOVA).

Interestingly, whereas loss of Kindlin-1 in the immunocompetent, FVB mice resulted in complete reduction in growth rate and clearance of the tumour (Fig. 24A-B), in the CD-1 nude immunocompromised mouse model, the growth rate was reduced but the tumour was not completely cleared (Fig. 24C-D). CD-1 nude mice lack T cells (229), which suggests that Kindlin-1 may regulate T cell responses.

Data from our group showed that *in vitro*, Met-1 Kin1-Null cells express IL-6 whereas Kin1-WT cells do not (Fig. 25A). This is important as Tregs actively supress the anti-tumour immune response (230). IL-6 is an important cytokine in pushing the differentiation of naïve CD4+ T cells away from regulatory T cells (Tregs) and towards T helper 7 (Th17) cells (Fig. 25B) (231). Data from our group also showed that tumours from Met-1 Kin1-Null mice have significantly reduced Tregs, which is demonstrated by significantly reduced levels of the transcription factor, FoxP3, a specific marker of Tregs (Fig. 25C) (230). In addition, *in vitro* conditioned media from Kin-1 Null cells caused naïve CD4+ T cells to differentiate into Th17 cells, which is demonstrated by higher levels of the transcription factor RoRgammaT, a marker of Th17 cells (Fig. 25D) (232). As IL-6 protein levels were significantly increased in Kin1-Null cells, we also examined IL-6 mRNA expression, however, IL-6 mRNA did not significantly differ between Kin1-Null and Kin1-WT cells suggesting that Kindlin-1 does not transcriptionally regulate IL-6 (Fig. 25E).

As differential tumour growth with Kindlin-1 loss in the immunocompetent and immunodeficient mouse models suggested that Kindlin-1 may regulate T cells, we

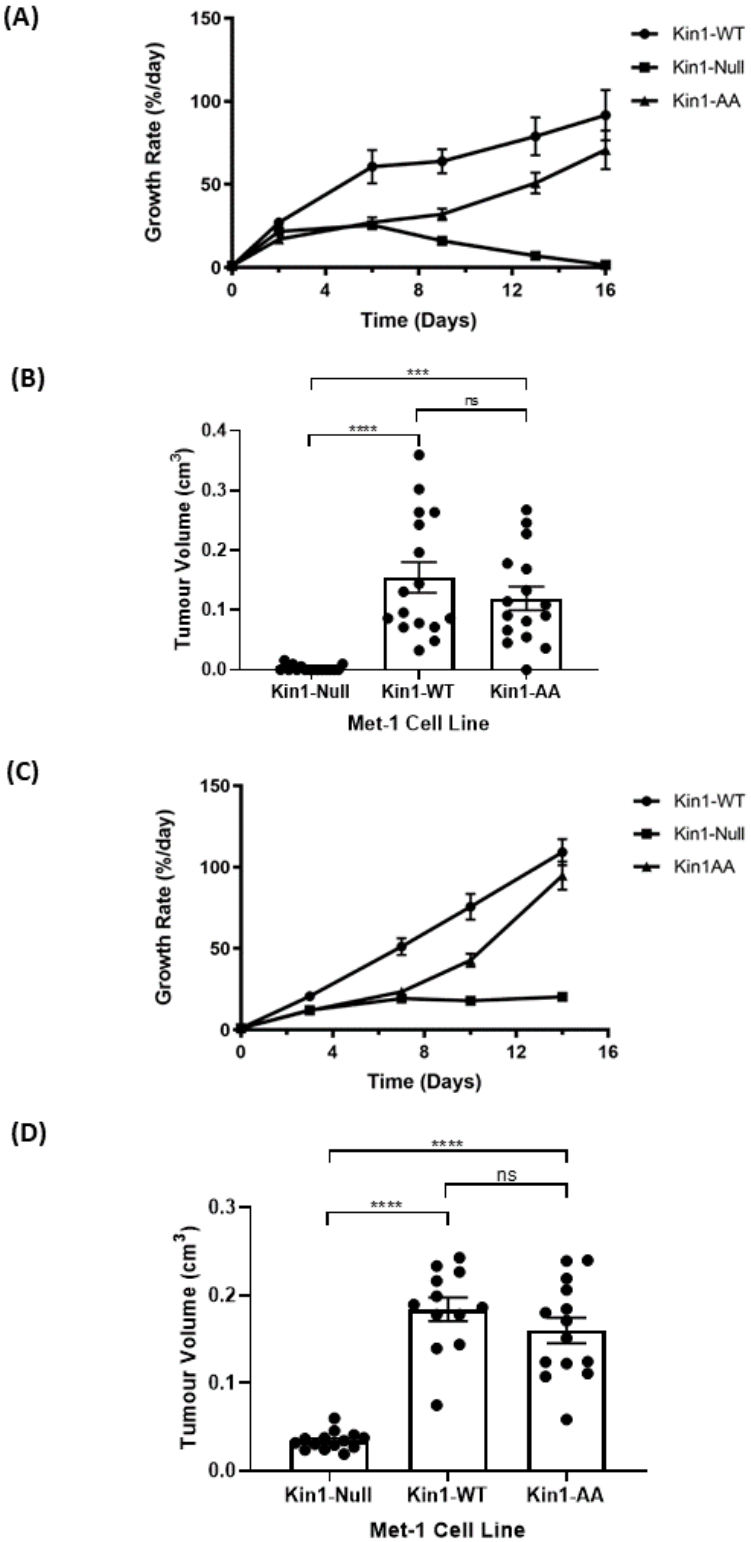


Figure 24. Tumour growth is significantly reduced with loss of Kindlin-1 in Met-1 cells in FVB and CD1 mice models. (A) Mean (±SEM) tumour growth of FVB mice inoculated with Met-1 Kin1-Null, Kin1-WT or Kin1-AA cells (n=16 tumours per group). (B) Mean (±SEM) tumour volume at day 16 of FVB mice inoculated with Met-1 Kin1-Null, Kin1-WT or Kin1-AA cells (n=16 tumours per group, ***p<0.001, ****p<0.0001, one-way ANOVA). (C) Mean (±SEM) tumour growth of CD-1 nude mice inoculated with Met-1 Kin1-Null, Kin1-WT or Kin1-AA cells (n=12 tumours for Kin1-WT cells and n=14 tumours for Kin1-

Null and Kin1-AA cells). (D) Mean (±SEM) tumour volume at day 14 of CD-1 nude mice inoculated with Met-1 Kin1-Null, Kin1-WT or Kin1-AA cells (n=12 tumours for Kin1-WT cells and n=14 tumours for Kin1-Null and Kin1-AA cells, ****p<0.0001, one-way ANOVA).

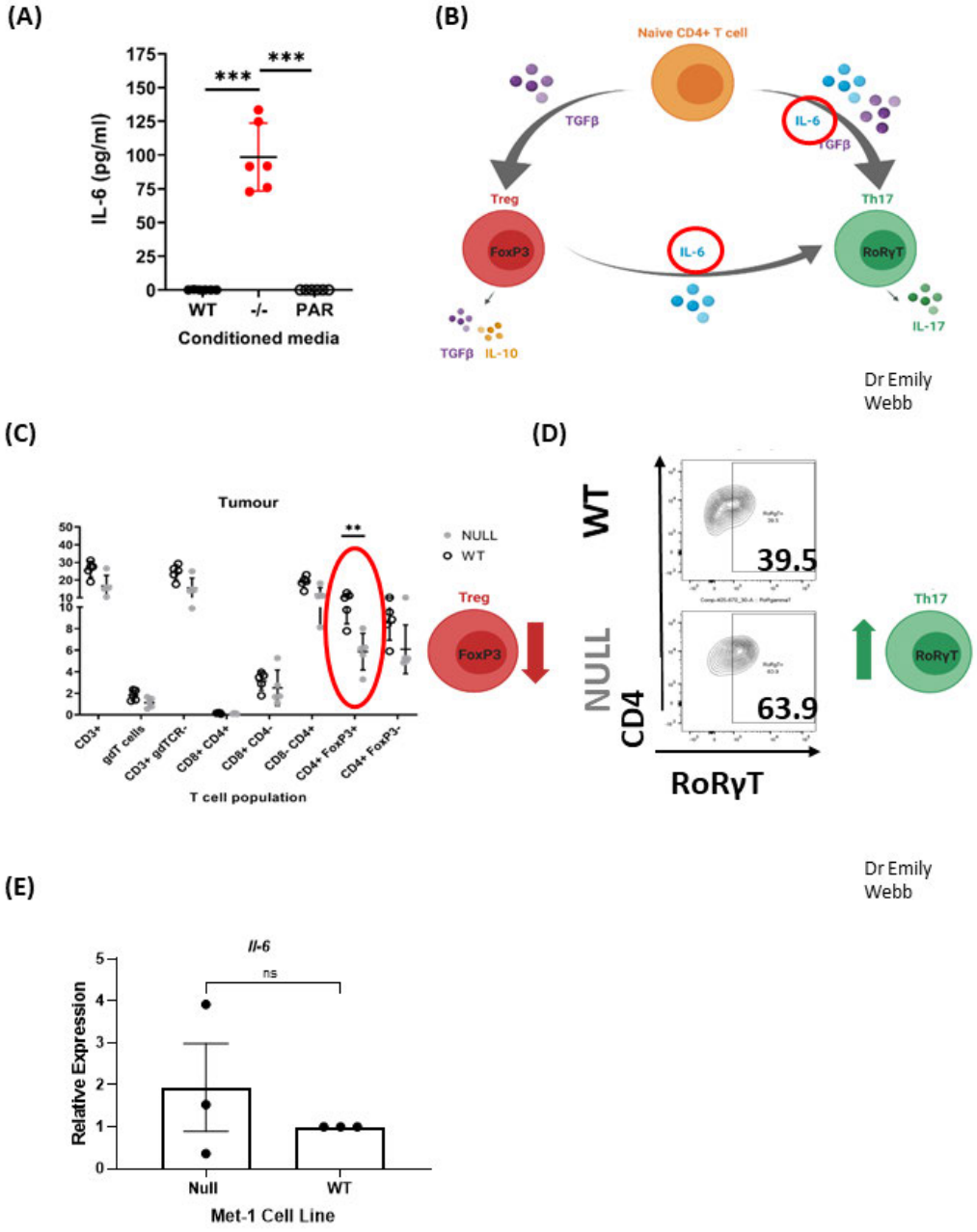
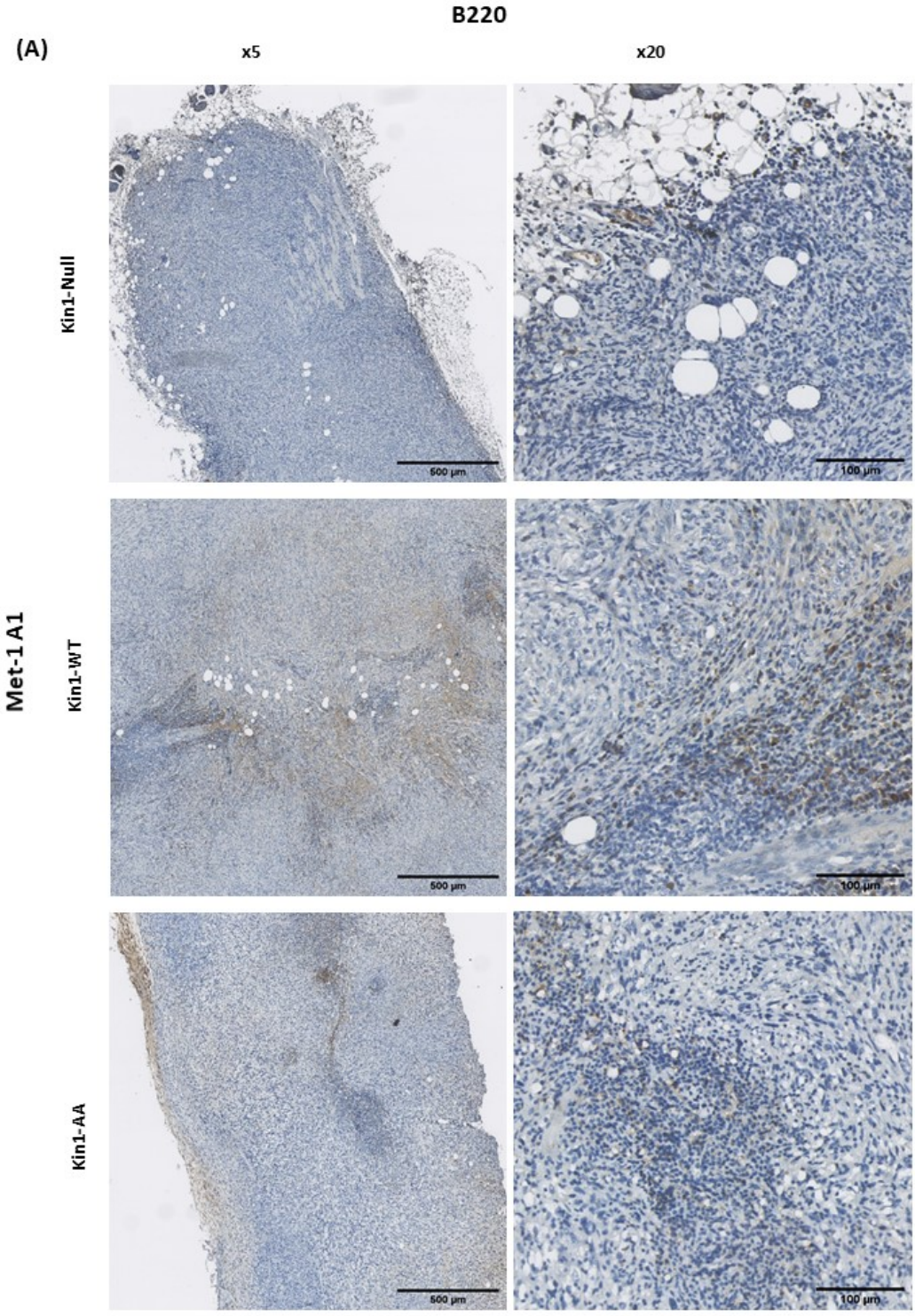


Figure 25. Met-1 Kin1-Null cells produce IL-6 and have an increased population of T helper 17 cells. (A) Quantitative analysis of IL-6 expression by ELISA assay in concentrated media of Met-1 Kin1-Null, Kin1-WT and parental cells. Combined data (average±SEM) from 6 independent experiments is shown for cell lines and a one-way ANOVA performed to test for statistical significance (***p<0.001). (Data from Emily Webb) (B) IL-6 is an important cytokine in pushing the differentiation of naïve CD4+ T cells

away from T regulatory (Tregs) cells and towards T helper 17 (Th17) cells. This is important as Tregs are regulatory T cells and actively supress the immune response to tumour cells. (Data from Emily Webb) (C) Quantitative analysis of T cell markers by flow cytometry in Met-1 Kin1-Null and Met-1 Kin-1 WT tumours. Combined data (average±SEM) from 5 tumours is shown for each cell line (**p<0.01). P values were obtained by a two-tailed Student's t test. (Schematic produced by Emily Webb) (D) Flow cytometry analysis of transcription factor RoRgammaT in CD4+ T cells in Met-1 Kin1-Null and Kin1-WT cells treated with Met-1 Kin1-Null conditioned media (Data from Emily Webb) (E) Expression of *Il-6* was analysed by qRT-PCR; values are mean ± SEM of triplicate experiments. Gene expression in Met-1 Kin1-Null cells was normalised to Met-1 Kin1-WT cells and a two-tailed Student's t test was performed to test for statistical significance.

performed IHC with the immune markers B220 and CD3 (Fig. 26). B220 is commonly used as a pan B cell marker in mice but is only expressed by a subset of B cells in humans (233). It may act as differentiation-specific marker in human B cells, marking mature naïve B cells (233). Tumours from Met-1 Kin1-WT and Kin1- AA cells had some diffuse B220 staining whereas, Kin1-Null tumours were largely negative for B220 (Fig. 26A). In order to look at the T cells, tumours were stained with CD3, which is a highly effective T cell marker as it is present at all stages of T cell development and is exclusive to the T cell lineage (234). Met-1 Kin1-WT and Kin1-AA tumours were mostly negative for CD3 staining compared to Kin1-Null tumours (Fig. 26B).

As data from the Met-1 model showed that Kindlin-1 regulated IL-6 at the protein level, we looked at IL-6 expression in the MDA-MB-231 model, however, *IL-6* did not significantly differ between the groups *in vitro* or *in vivo* (Fig. 27). To further investigate if Kindlin-1 has a role in regulating T cells, we also performed IHC with immune markers in the tumours from the CD1 nude mice to evaluate if Kindlin-1 affects the immune system in a human model (Fig. 28). The mouse tissues (Fig. 26) were run alongside the human tissues as a positive control, however, tumours from shRNA Kin-1 –dox and +dox stained negatively for B220 (Fig. 28A) and CD3 (Fig. 28B) despite having cross species reactivity for both mouse and human tissues.


B220
(A)
x5
x20
Met-1 A1
Kin1-Null
Kin1-WT
Kin1-AA
500 µm
100 µm
500 µm
100 µm
500 µm
100 µm

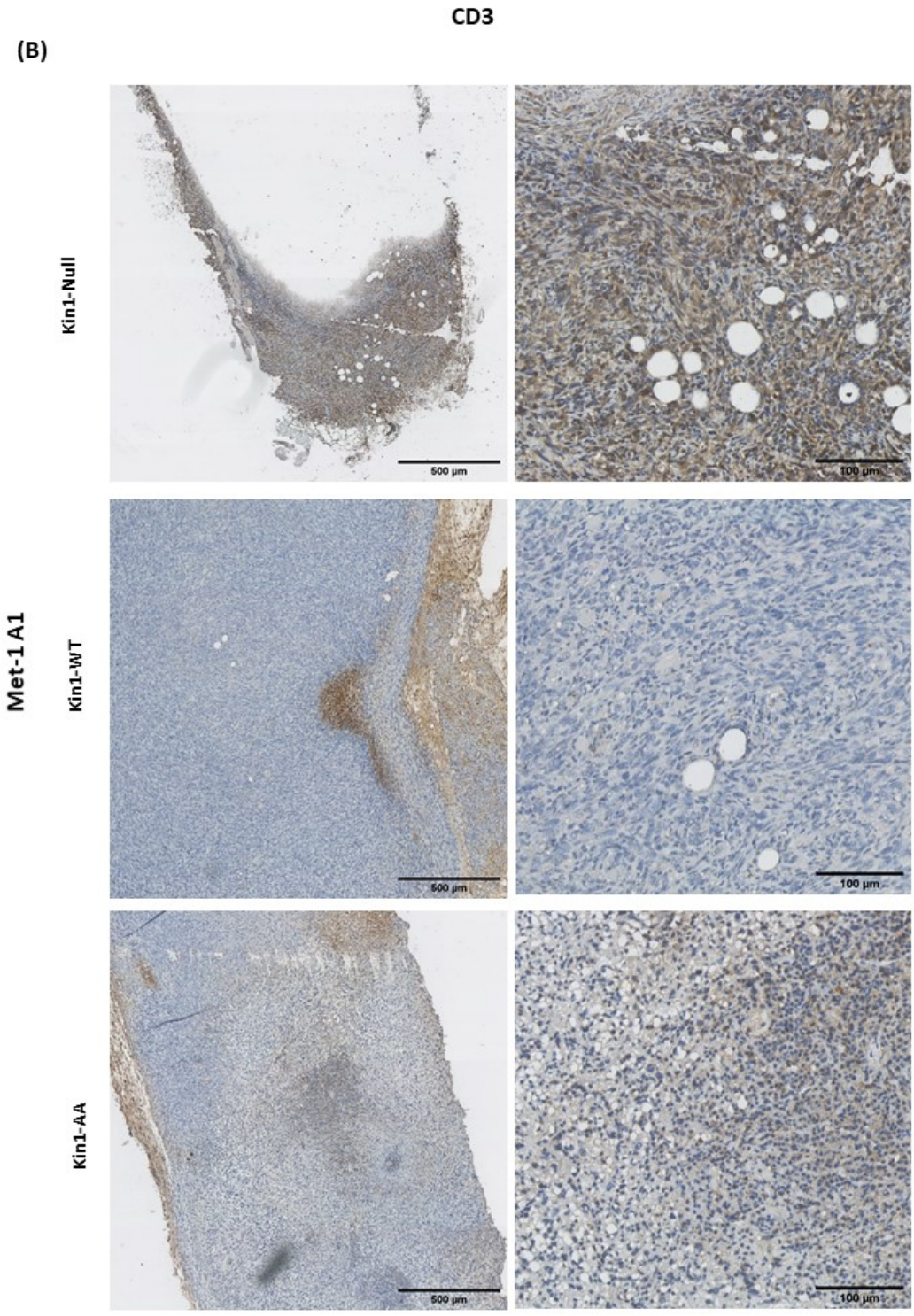


Figure 26. Presence of immune markers in tumours from FVB mice inoculated with Met-1 cells. Representative images of immunohistochemistry staining of (A) B220 and (B) CD3 in Met-1 Kin1-Null, Kin1-WT and Kin1-AA cells. Objective magnification: 5x (left) and 20x (right). Scale bar represents 500µm (left) and 100µm (right).

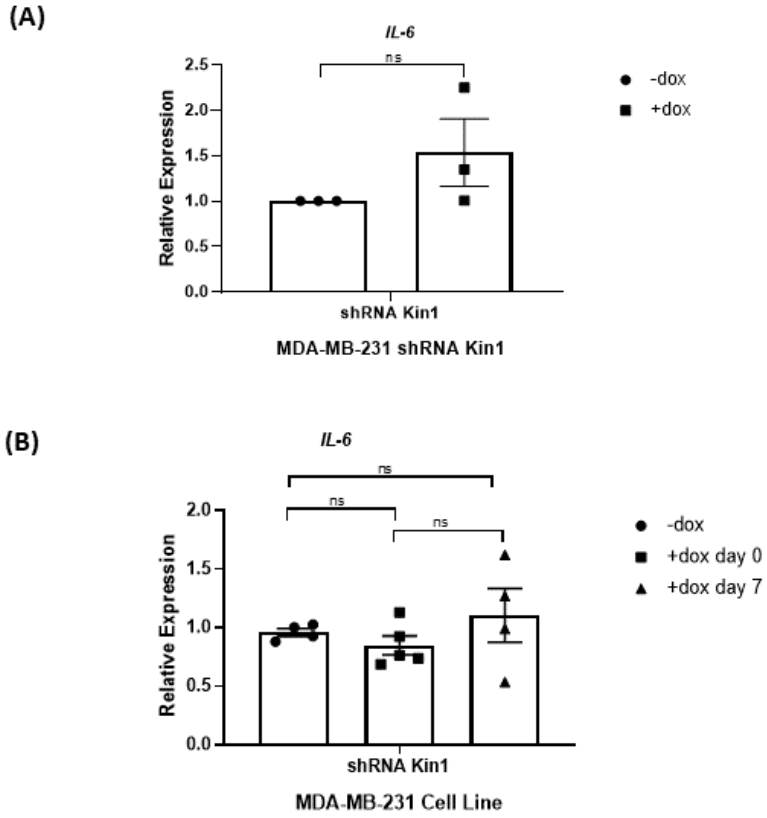


Figure 27. Analysis of *IL-6* expression in the MDA-MB-231 model. (A) Analysis of *IL-6* expression in MDA-MB-231 shRNA Kin1 cells –dox or +dox cells. Expression of *Il-6* was analysed by qRT-PCR; values are mean ± SEM of triplicate experiments. Gene expression in +dox cells was normalised to –dox cells and a two-tailed Student's t test was performed to test for statistical significance. (B) Analysis of *IL-6* expression in MDA-MB-231 shRNA Kin1 cells –dox or +dox tumours from SCID mice. Expression was analysed by qRT-PCR; values are mean ±SEM of 4 tumours for –dox cells, 5 tumours for +dox day 0 cells and 4 tumours for +dox day 7 cells. Gene expression was normalised to -dox cells. (**p*<0.05,***p*<0.01, one-way ANOVA).

3.8 Characterisation of a Kindlin-1 antibody generated by Movarian-Biotech

Due to problems with the specificity of the Abcam Kindlin-1 antibody (Fig. 24), a polyclonal Kindlin-1 antibody was designed by our group and generated by Movarian-Biotech. The epitopes used to generate the Kindlin-1 antibody were designed to react with both mouse and human Kindlin-1 sequences at the C-terminus (Fig. 29A). The Abcam antibody is a polyclonal antibody that has been raised to react with mouse and human Kindlin-1 sequences at the C-terminus (Fig. 29B). The Movarian Biotech and Abcam Kindlin-1 antibodies were compared by western blot using the Met-1 cell

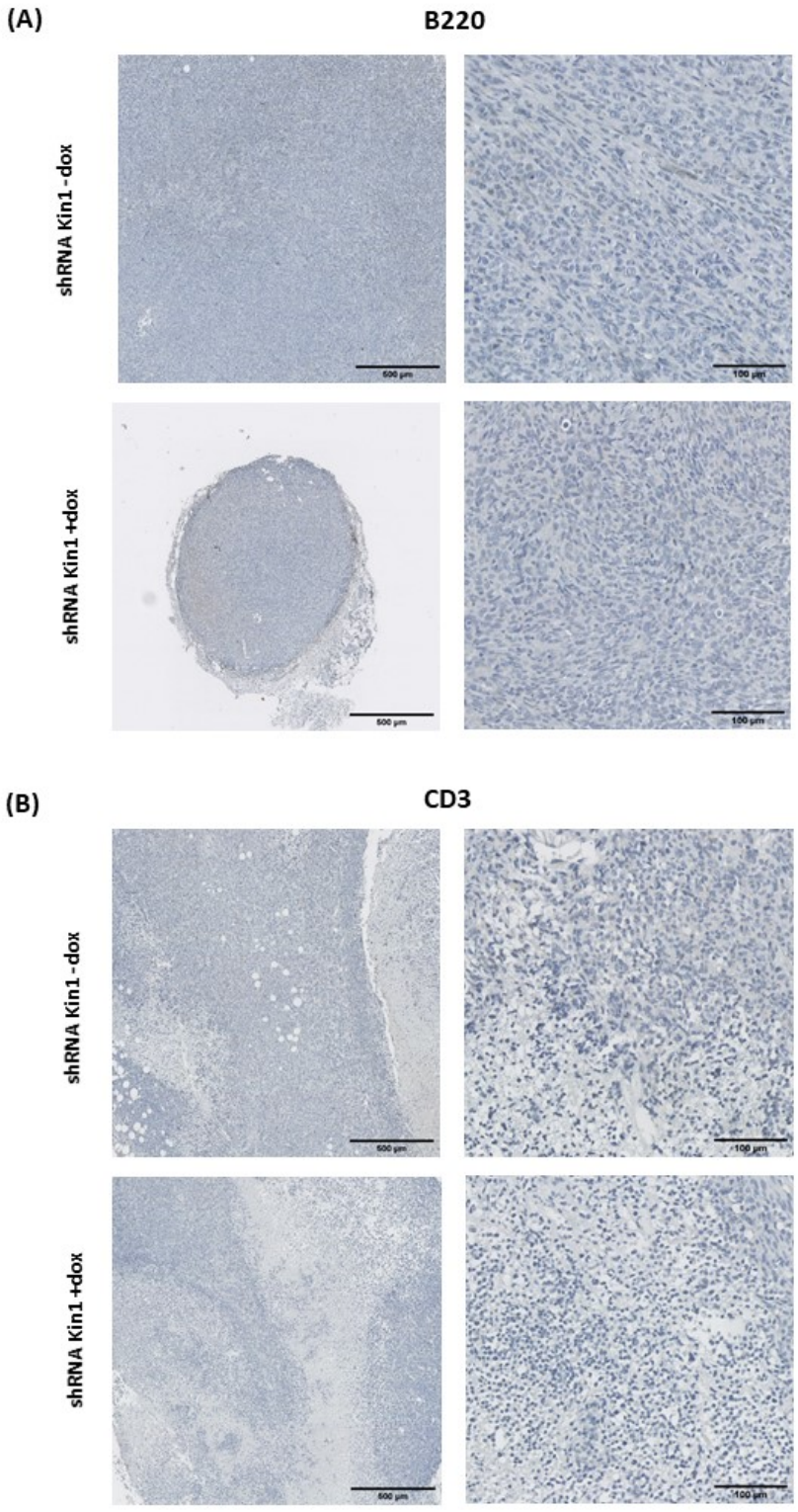


Figure 28. Presence of immune markers in tumours from CD-1 nude mice inoculated with MDA-MB-231 shRNA Kin1 cells. Representative images of immunohistochemistry staining of (A) B220 and (B) CD3 in MDA-MB-231 shRNA Kin1 cells –dox and +dox subcutaneous tumours. Objective magnification: 5x (left) and 20x (right). Scale bar represents 500µm (left) and 100µm (right).

(A)

```
Kindlin-1_HUMAN  QNVAQMPLVEAKLRFIQAWQSLPEFGLTYYLVRFKGSKKDDILGVSYNRLIKIDAATGIP 600
Kindlin-1_MOUSE  HNVAQMPLVEAKLQFIQAWQSLPEFGLTYYLVRFKGSKKDDILGVAYNRLIRIDAVTGIP 600
peptide1         ------------------------------------------------------------
peptide2         ------------------------------------------------------------

Kindlin-1_HUMAN VTTWRFTNIKQWNVNWETRQVVIEFDQNVFTAFTCLSADCKIVHEYIGGYIFLSTRSKDQ 660
Kindlin-1_MOUSE VTTWRFANMKQWNVNWEIRQVAIEFDQNVSIAFTCLSADCKIVHEYIGGYIFLSTRSKDQ 660
peptide1        ------------------------------------------------------CSTRSKDQ 8
peptide2        ------------------------------------------------------------

Kindlin-1_HUMAN NETLDEDLFHKLTGGQD 677
Kindlin-1_MOUSE NETLDEDLFHKLTGGQD 677
peptide1        NETLDE---------- 14
peptide2        -CTLDEDLFHKLTGGQD 16
```

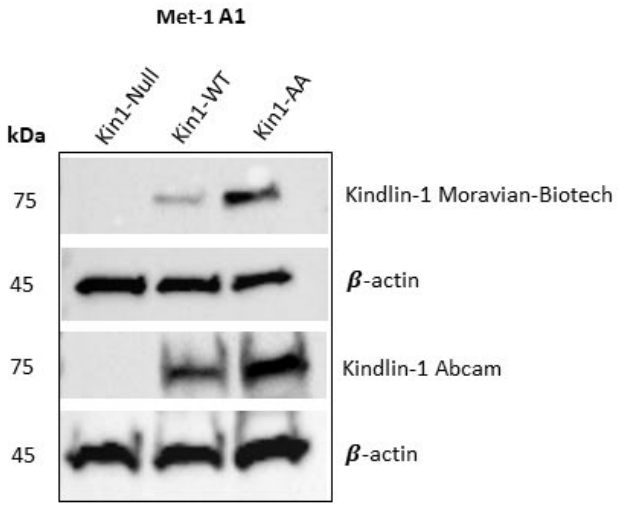
(B)

```
Kindlin-1_HUMAN  QNVAQMPLVEAKLRFIQAWQSLPEFGLTYYLVRFKGSKKDDILGVSYNRLIKIDAATGIP 600
Kindlin-1_MOUSE  HNVAQMPLVEAKLQFIQAWQSLPEFGLTYYLVRFKGSKKDDILGVAYNRLIRIDAVTGIP 600
peptide          ------------------------------------------------------------

Kindlin-1_HUMAN VTTWRFTNIKQWNVNWETRQVVIEFDQNVFTAFTCLSADCKIVHEYIGGYIFLSTRSKDQ 660
Kindlin-1_MOUSE VTTWRFANMKQWNVNWEIRQVAIEFDQNVSIAFTCLSADCKIVHEYIGGYIFLSTRSKDQ 660
peptide         ------------------------------------------------------CKDQ 4

Kindlin-1_HUMAN NETLDEDLFHKLTGGQD 677
Kindlin-1_MOUSE NETLDEDLFHKLTGGQD 677
peptide         NETLDEDLFHKLTGGQD 21
```

(C)



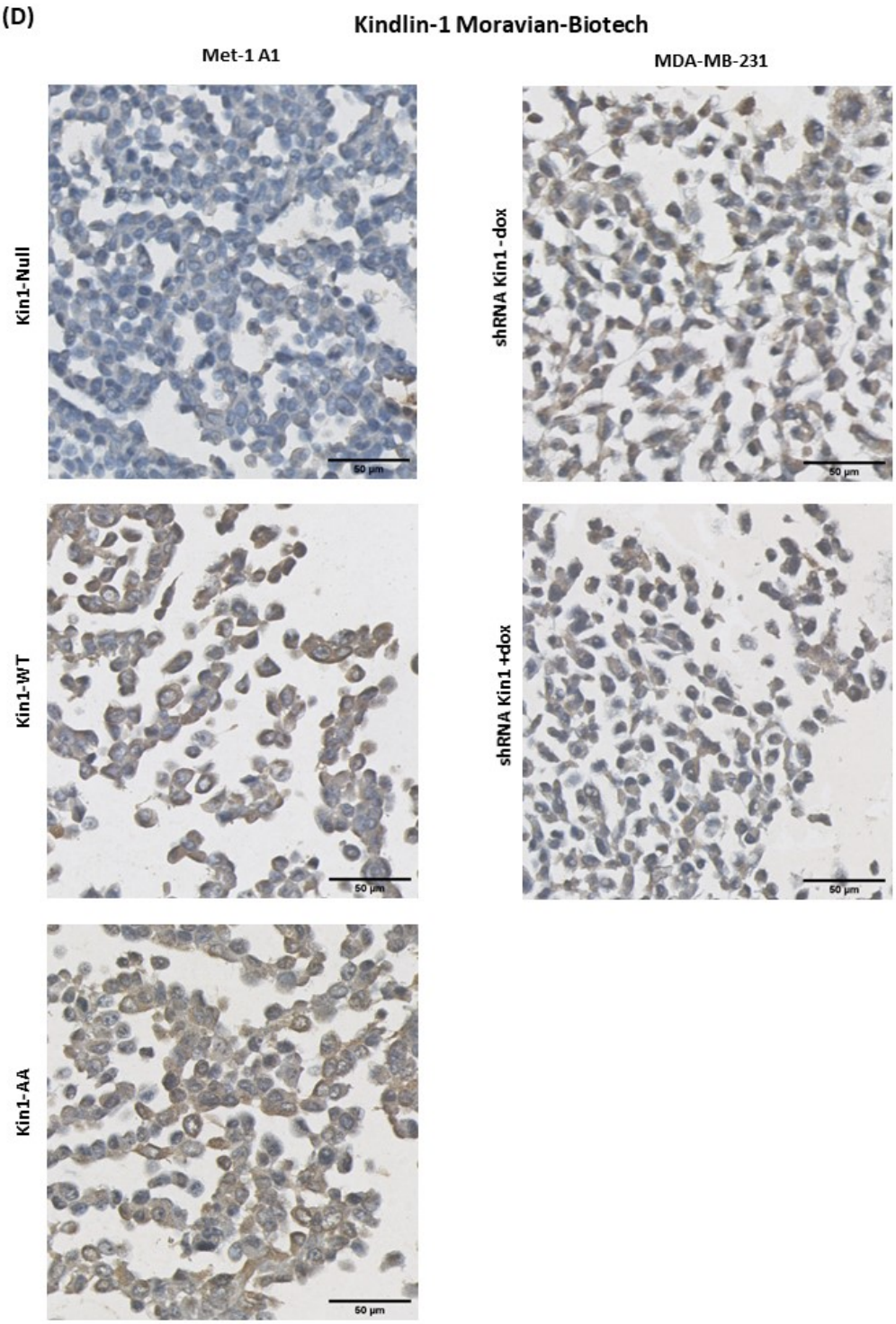


Figure 29. Evaluation of Kindlin-1 antibody generated by Movarian-biotech. (A) Alignment of epitopes raised against the Kindlin-1 sequence for generation of the Movarian-Biotech Kindlin-1 antibody (B) Alignment of the epitope raised against the Kindlin-1 sequence for the generation of the Abcam Kindlin-1 antibody (C) Western blot analysis for Kindlin-1 expression in Met-1 A1 Kin1-Null, Kin1-WT and Kin1-AA cells using the Movarian-Biotech and Abcam Kindlin-1 antibodies. $\boldsymbol{\beta}$-actin was used as a loading control. (D) Immunohistochemistry staining of Kindlin-1 protein expression in Met-1 A1 Kin1-Null, Kin1-WT and Kin1-AA cells and MDA-MB-231 shRNA Kin1 cells –dox and +dox cells. Objective magnification: ×10. Scale bar represents 50µm.

line (Fig. 29C). Both antibodies specifically bound to Kindlin-1 in Met-1 Kin1-WT and Kin1-AA cells and a band was not detected in Kin1-Null cells (Fig. 29C). Immunohistochemistry staining of Met-1 cell pellets using the Movarian Biotech antibody demonstrated that the antibody stained Met-1 Kin1-WT and Kin1-AA cells but did not stain cells from Met-1 Kin1-Null cell line (Fig. 29C). The Movarian Biotech antibody stained both MDA-MB-231 shRNA Kin1 –dox and +dox cells, suggesting that the antibody might non-specifically stain human cells (Fig. 29D).

3.9 Discussion

3.9.1 Regulation of mammosphere formation by Kindlin-1

As we previously showed that Kindlin-1 regulates *Tnc* (185) and tenascin-C has a defined role in regulating cancer stem cell self-renewal through the regulation of the Notch and Wnt pathways via *MSI1* and *LGR5,* respectively (205), we sought to establish if Kindlin-1 has a role in regulating stem cell activity. The *in vitro* mammosphere assay can be used to define early stem cell or early progenitor activity through the generation of primary mammospheres and investigate stem cell self-renewal through the generation of secondary mammospheres (212). The first results from the mammosphere assay suggested that *FERMT1* expression significantly increased secondary formation of mammospheres and therefore, Kindlin-1 may have a role in cancer stem cell self-renewal (Fig. 13). Previous data from the Brunton group using the PyMT murine model of breast cancer, showed that loss of Kindlin-1 has an effect on tumour initiation, which is suggestive of an effect on stem cells. Furthermore, integrins, including β1 integrin, which Kindlin-1 is known to bind, have been associated with the differentiation (235,236) and self-renewal properties of stem cells (237,238). For example, it has been shown that in PyMT mice, β1 integrin is necessary for the generation or amplification of CD24highCD29lowCD61high cancer cells (239,240). Collectively, this suggests that Kindlin-1 may have a role in stem cell signalling, however, in subsequent experiments using the mammosphere

assay, we did not see the same effect on mammosphere self-renewal with loss of Kindlin-1 and saw a large variation between the replicates of each experiment (Compare Fig. 13C with Fig. 16C and 16D).

There are a number of methodological difficulties with the *in vitro* mammosphere assay and several limitations to the assay, which may affect accuracy of results and account for the variation in results we obtained when performing the assay. The key principle of the mammosphere assay is that each mammosphere is derived from a single cell and is therefore clonal. Fusion or aggregation of mammospheres can either occur as a result of plating density or movement of plates during the culture period, or when examining cultures under the microscope and this can result in problems with interpretation of results. Furthermore, cell loss can happen when dissociating primary mammospheres for generation of secondary mammospheres, which can also affect the results produced. Additionally, the protocols that are used by different groups vary, which can mean that the results are inconsistent. The mammosphere assay performed in this project involved counting the number of mammospheres in the whole well, whereas some research groups take several random images of the wells and perform calculations using the number of mammospheres per field, which may not be a true representation of the results. We have analysed and displayed the primary and secondary mammospheres as mammosphere forming efficiency percentage and mammosphere self-renewal percentage, whereas some groups present the data as spheres/10,000 cells or the number of mammospheres per field, which makes it difficult to compare the results from different studies.

As well as mammosphere assays, colony forming assays have been used as an *in vitro* quantitative technique to study cancer stem cells. The colony forming assay is an agarose-based assay that examines the capability of a single cell to grow into a large colony through clonal expansion (241). This assay could be a useful alternative to the mammosphere assay and may provide a solution to some of the issues with the mammosphere assay such as aggregation of mammospheres due to plate movement.

In addition, to explore if the conflicting results that we obtained from the mammosphere assay are a result of the variability of the assay, comprehensive analysis of stem cells markers *in vitro* could be carried out as only a small selection of these markers were evaluated in this study (Fig. 14). The cell surface markers of breast CSCs have been determined through *in vivo* experiments in which human breast cancer cells are grown in immunocompromised mice. One study showed that the oncogenic subpopulation expressed $CD24^-/CD44^+$ surface markers and only this minority of tumour cells could initiate new tumours (25). Therefore, it would be beneficial to analyse $CD24^-/CD44^+$ cell populations in the mammospheres by FACs in order to detect whether the mammosphere assay selects for breast CSCs.

Although *in vitro* mammosphere assays may demonstrate the capability of cells to display stem cell traits, they are not a read out of *in vivo* stem cell frequency as they do not capture the complexity of CSC formation or behaviour *in vivo* (242). Mammosphere assays lack the niche components such as the immune and stromal components that can be provided by xenograft transplantation experiments (243). Lineage tracing experiments rely on using markers to identify the CSC subset and enable the dynamic *in vivo* formation of CSCs to be traced (242). Thus, *in vivo* transplantation paradigms or *in vivo* lineage tracing experiments are required to complement the mammosphere assay and provide a more informative view of the CSC properties of a cell line.

We also explored breast stem cell activity in a human model of breast cancer but despite obtaining sufficient knockdown of Kindlin-1 in a human breast cancer model (Fig. 17C-D), we did not observe an effect on mammosphere formation in the MDA-MB-231 model (Fig. 18A) as observed in the initial mammosphere experiment in the Met-1 model (Fig. 13C). Although mammosphere self-renewal percentage was lower with knockdown of Kindlin-1, mammosphere self-renewal was not significantly lower (Fig. 18A). We saw some variation between the replicates of the mammosphere self-renewal percentage in MDA-MB-231 shRNA Kin1 mammospheres and this perhaps

made it difficult to identify small changes, however, the expression of *TNC* was also unaltered upon Kindlin-1 knockdown (Fig. 18B), which may correlate with the non-significant mammosphere self-renewal result that we observed in the MDA-MB-231 model. This suggests that the phenotype observed in the Met-1 model is not universal and cell type specific.

A fundamental hypothesis of the CSC theory is that CSCs drive the formation of metastases. One area of research that explicitly implicates CSCs as metastasis-initiating cells which, in association with the metastatic niche, generate metastatic nodules, is work on tenascin-C in breast CSCs by Oskarsson *et al.* (205). We have shown that Kindlin-1 expression correlates with *TNC* expression in a human breast cancer cell line that was used by Oskarsson *et al.* (205) for their research on tenascin-C and pulmonary breast cancer metastasis (Fig. 21A-B). This finding correlates with previous data from the Brunton group, which showed a strong correlation between *FERMT1* and *TNC* and the lung metastatic capacity of MDA-MB-231 variants (185). We used CRISPR-cas9 technology to knock out Kindlin-1 in the lung metastatic variant of the human breast cancer cell line (Fig. 19C), however, we were unable to confirm knockout in single cell clones due to issues with the specificity of a batch of the Abcam Kindlin-1 antibody by western blot. A Kindlin-1 antibody was designed by the Brunton group and generated by Moravian Biotech in order to overcome this problem (Fig. 29), however, even though specificity of antibody for Kindlin-1 was demonstrated by western blot (Fig. 29C), we did not have time to pursue this further. As Kindlin-1 may be important for *TNC* expression in this model, future work to generate a Kindlin-1 knockout cell line would be a useful tool to explore if loss of Kindlin-1 has an effect on mammosphere formation and stem cell signalling.

As previous *in vivo* data from our group demonstrated that loss of Kindlin-1 significantly reduced metastatic growth in the lungs of mice and we have shown here that *Tnc* is significantly increased in Met-1 mammospheres that express Kindlin-1 (Fig. 14) and that Kindlin-1 expression correlates with *TNC* expression in a more lung

metastatic human breast cancer cell line (Fig. 19A-B), this strongly suggests that Kindlin-1 has a role in stem cell regulation and further research needs to be carried out to explore the role of Kindlin-1 in regulation of stem cells.

3.9.2 The role of Kindlin-1 in the regulation of signalling pathways that control stem cells.

Expression of cancer cell-derived tenascin-C has been shown to promote breast cancer pulmonary metastasis by positively regulating two stem cell signalling pathways; Notch and Wnt (205) and as we found Kindlin-1 regulates *Tnc* expression (185), we explored expression of stem cell markers in the Met-1 mammospheres (Fig. 14). Kindlin-1 has been previously reported to regulate the proliferation and differentiation of cutaneous epithelial stem cells through two different mechanisms; by activating the release of TGF-β via αvβ6 integrin and by inhibiting Wnt-β-catenin signalling through regulation of the expression of Wnt ligand via a mechanism that is independent of integrin (156). Our results suggested that loss of Kindlin-1 does not affect the Wnt pathway but instead suggested that Kindlin-1 may regulate stem cell self-renewal through the Notch pathway by regulating expression of a positive regulator of the Notch pathway, *Msi1* (Fig. 14). As expression of tenascin-C in breast CSCs was previously linked to enhanced expression of MSI1 and regulation of the Notch pathway (205), we chose to investigate the Notch pathway. We did not follow up the implications of the differential expression of *Nanog,* which was significantly decreased in Kin1-WT secondary mammospheres (Fig. 14). Nanog is a master transcription factor that controls self-renewal and also, pluripotency of stem cells, however, it has been previously reported that disruption of *TNC* expression in secondary mammospheres does not affect expression of *NANOG* (205). In addition, it has been shown that overexpression of Nanog in adult mammary tissues of a transgenic mouse model is not sufficient to induce tumorigenesis (244).

We expected that inhibition of Notch signalling in both Met-1 Kin1-Null and Kin1-WT cells would result in decreased mammosphere formation. However, inhibition of Notch signalling did not negatively affect mammosphere formation in Kin1-Null or Kin1-WT cells (Fig. 16B,D) suggesting that the Notch pathway is dispensable for the induction of pluripotent stem cells in this model. Notably, studies using the GSI DBZ were in human breast cancer cell models, and these studies have shown that with addition of the inhibitor, the mammospheres generated are much smaller (228). The size of the Met-1 mammospheres generated in the presence of the inhibitor were not affected (Fig. 16A), further strengthening the argument that the Notch pathway is not essential for stem cell signalling in this murine breast cancer model.

In glioma, it has been reported that the intracellular domain of the Notch2 receptor can also bind to the *Tnc* promoter and as a result, a positive feedback loop may be established with *Tnc* expression and Notch signalling (245). We therefore expected Notch inhibition may decrease expression of *Tnc*, however, *Tnc* expression was not significantly altered with Notch inhibition in Kin1-Null and Kin-1 WT mammospheres (Fig. 16C). In addition, a higher level of cleaved Notch1 was found in Kin1-Null cells compared to Kin1-WT cells, suggesting that expression of Kindlin-1 inhibits cleavage of Notch1 (Fig. 15B). Our results appear to contradict the literature as we find significantly increased expression of *Msi1,* a positive regulator of the Notch pathway, and tenascin-C in Kin1-WT cells (Fig. 14) yet, we find less expression of cleaved Notch in Kin1-WT cells compared to Kin1-Null cells (Fig. 15B). Our results suggest that the Notch pathway does not have a general effect on mammosphere formation or expression of *Tnc* in this cell model. Kindlin-1 may regulate formation of mammospheres in the secondary generation through regulation of tenascin-C expression; however, the results suggest that Kindlin-1 does not engage in the Notch pathway to support the fitness of tumour-initiating cells in the Met-1 model.

A multitude of signalling pathways which control normal stem cell self-renewal and differentiation to generate progeny that populate a tissue have been implicated in

CSC signalling. When dysregulated following mutation; Janus-activated kinase/signal transducer and activator of transcription (JAK/STAT) (246), WNT (247,248), Sonic Hedgehog (SHH) (249,250), Notch (251–253), phosphatidylinositol 3-kinase/phosphatase and tensin homolog (PI3K/PTEN) (254,255), nuclear factor-κB (NF-κB) (256) and BMI1 (257,258) have all been linked to neoplastic proliferation in breast cancer. Therefore, it is possible that Kindlin-1 has a role in regulating of stem cells via a stem cell signalling pathway other than Notch. We have shown that Kindlin-1 regulates the protein levels of the cytokine IL-6 (Fig. 13A) and as this protein has been linked to NF-κB signalling (259), this could be an avenue to explore next.

3.9.3 Regulation of tumour formation by Kindlin-1

Previous research has shown that Kindlin-1 is a mediator of pulmonary breast cancer metastasis (185,186,207,209) and breast tumorigenicity (155,186). Clinical data has shown that kindlin-1 potentially mediates lung metastasis in breast cancer (186) and has also highlighted a potential role for Kindlin-1 in primary breast tumorigenesis as breast tumour tissues had significantly upregulated Kindlin-1 protein levels compared to normal adjacent breast tissue (155). Genes that promote metastasis are generally not associated with primary tumour growth, however, our data is consistent with previous research showing that Kindlin-1 loss decreases primary tumour growth (Fig. 24). Sin *et al.* (186) showed that silencing of Kindlin-1 using an orthotopic mouse model prevented tumour growth and lung metastasis, however, these effects could be linked. As this is a spontaneous metastasis model, decreased metastasis could be a result of the reduced primary tumour growth observed with the suppression of Kindlin-1. Experimental metastases assays bypass this issue as cells are inoculated via the tail vein of mice and cells that survive the circulation, accumulate in the lungs and form micrometastases. Previous data from the Brunton group showed that metastatic burden was reduced with loss of Kindlin-1 in an experimental metastasis assay (185). In this study, in addition to the experimental metastasis model, the Brunton group used a PyMT genetically engineered mouse model of Kindlin-1 loss.

This model had no effect on primary tumour growth but delayed tumour onset and reduced metastasis demonstrating that the different models can give rise to different effects (185). Genetically engineered mouse models have spontaneous autochthonous tumour development, which may be important. Tumours from genetically engineered mouse models of metastasis develop in a normal context and in a system that possesses a functional immune system (260).

Sin *et al.* (186) also investigated overexpression of Kindlin-1 and showed that these cells had increased proliferation, clonogenicity, and invasion *in vitro.* These are hallmarks that are important for initiation and progression of primary tumours from metastasis (19,56). Ectopic Kindlin-1 expression was also shown to induce EMT and this was associated with increased TGF-β signalling (186). Several TGF-β target genes that have previously been linked to breast cancer progression were upregulated in Kindlin-1 overexpressing cells (186). TGF-β signalling has been shown to promote lung metastasis formation in several transgenic mouse models suggesting that mechanistically, regulation of TGF-β signalling by Kindlin-1 may promote breast cancer progression (186,261,262). It has been speculated that Kindlin-1-mediated activation of αvβ6 integrin is responsible for increased TGF-β signalling in breast cancer as Kindlin-1 has been shown to activate αvβ6 integrin and promote TGF-β signalling in keratinocytes (131,171).

We also demonstrate that activation of integrin by Kindlin-1 is required for tumour initiation in primary tumours as loss of Kindlin-1-integrin binding caused a distinct lag in tumour growth (Fig. 24A,C). Once the tumour has been established, tumours expressing a mutant Kindlin-1 that is unable to bind integrin grow to a similar volume as tumours that express Kin1-WT (Fig. 24A,C). This provides evidence that after tumour initiation has occurred, the interaction of Kindlin-1 with integrin is no longer necessary for tumour growth (Fig. 24A,C) and suggests that Kindlin-1 has roles independent of integrin that are important for tumour growth. Kindlin-1 has been reported to have an integrin-independent role in Wnt signalling (171). It has been

suggested that Kindlin-1 may inhibit Wnt signalling at the transcriptional level by retaining transcriptional cofactors in the cytoplasm (171). As the integrin independent roles of Kindlin-1 are largely unknown and remain entirely unexplored in breast cancer, this study further highlights that these roles should be further scrutinised.

To our knowledge, this is the first study to show evidence that the immune system impacts on the Kindlin's ability to regulate tumour growth. Our results demonstrate that loss of Kindlin-1 in an immunocompetent mouse model results in complete clearance of the tumour (Fig. 24A-B), whereas loss of Kindlin-1 in an immunocompromised mouse model that has a reduced amount of T cells (229), does not result in complete clearance of the tumour by the immune system (Fig. 24C-D). We show that mechanistically, loss of Kindlin-1 post-transcriptionally upregulates production of the cytokine IL-6 (Fig. 25A,E), which causes differentiation of naïve CD4+ T cells into Th17 cells or differentiation of Tregs into Th17 cells (Fig. 25C-D). IL-6 is responsible for moderating the balance between IL-17 secreting Th17 cells and Treg cells, which secrete TGFβ and IL-10 (Fig. 25B) (reviewed in (263)). IL-6 promotes the generation of Th17 cells from naïve CD4+ T cells together with TGFβ or contrastingly, inhibits generation of Tregs by TGFβ (Fig. 25B). Tregs are responsible for suppressing the anti-tumour immune response and stimulating immunological ignorance to tumour cells by limiting effector T cell responses (reviewed in (264)). Loss of Kindlin-1 decreases the numbers of Tregs in tumours (Fig. 25C) and increases the population of Th17 cells (Fig. 25D). Th17 cells produce the pleiotropic cytokine IL-17, which in turn activates secretion of numerous pro-inflammatory cytokines and chemokines and results in tissue inflammation (reviewed in (265)). IL-17 has also be shown to induce neutrophil and macrophage recruitment and this has been shown to protect against microbes (266). Some studies have shown that Th17 cells can promote tumour growth but there is also evidence from murine tumour models that Th17 cells have anti-tumour effects, which correlates with our data. Th17 cells have been shown to eradicate tumour cells in a mouse model of advanced B16 melanoma

(267) and also recruit immune cells and anti-tumour immune responses by triggering cytolytic CD8+ T cell responses (268). In addition, Th17 cells have been shown to positively predict patient outcome by activating the Th1-type chemokines CXCL9 and CXCL10 in ovarian cancer patients, which recruits effector T cells (269). Th17 cells have also been shown to promote differentiation of CD4+ T cells into effector T cells, which effectively destroy tumour cells in lung cancer (270).

Staining suggested that tumours from Met-1 Kin1-Null cells had more T cells, compared to Kin1-WT tumours, however, Kin1-Null cell tumours were much smaller than Kin1-WT, which may account for this finding (Fig. 26B). In line with the literature, our results suggest that loss of Kindlin-1 promotes secretion of IL-6, which leads to differentiation of naive CD4+ T cells into Th17 cells and this has a protective anti-tumour function. In future studies, an IL-6 inhibitor could be used in mice inoculated with Met-1 Kin1-Null cells to establish if this leads to a tumour growth phenotype similar to Met-1 Kin1-WT cells. Furthermore, effector T cell populations could be investigated in the tumours.

We also investigated Kindlin-1 loss in a human breast cancer model and interestingly, we did not observe an effect on metastases formation in the lungs of mice inoculated with the MDA-MB-231 shRNA-Kin1 cells via the tail vein (Fig. 20A-B), contrary to what has been reported in the literature and what we have previously reported in the Met-1 model (185,186,207,209). It has been reported that Kindlin-1 mRNA transcripts are upregulated in the basal B subtype of breast cancer (186) and therefore, as MDA-MB-231 cell line is a basal B, TNBC cell line, we would expect loss of Kindlin-1 to reduce tumorigenesis. However, our results suggest that knockdown was not achieved as we found expression of Kindlin-1 in mice receiving the doxycycline diet (Fig. 20C). It is possible that there may have been an issue with the doxycycline diet in this experiment as we were able to confirm that *FERMT1* expression was significantly reduced in a subsequent experiment where mice were subcutaneously inoculated with the MDA-MB231 shRNA-Kin1 cells using fresh doxycycline diet (Fig. 21C).

However, the subcutaneous tumours with Kindlin-1 knockdown also stained positive for Kindlin-1 by IHC (Fig. 22), suggesting that the Kindlin-1 antibody non-specifically stains tissues. As Kindlin-1 expression is not completely abrogated in this model, some Kindlin-1 staining may occur in tumours from mice that received doxycycline diet and perhaps the concentration of the antibody needs to be optimised further for IHC use.

Interestingly, our initial experiment evaluating Kindlin-1 loss in primary tumour growth using the MDA-MB-231 model in CD1 nude mice showed a significantly decreased final tumour volume with Kindlin-1 loss (Fig. 21B). This result suggested that Kindlin-1 reduces tumour burden in a human and mouse model *in vivo.* Notably, in an SCC model of Kindlin-1 loss, loss of Kindlin-1 has the opposite effect on tumour growth compared to the breast cancer models discussed here and loss of Kindlin-1 significantly increases tumour volumes (210). It is possible that this is due to the different driver mutations that promote cancer development in different tumour types. Because of time restraints the tail vein experiment using new doxycycline diet was not revisited and we decided to focus on the subcutaneous data as we had interesting results with the Met-1 model.

Intriguingly, a subsequent subcutaneous tumour growth experiment in SCID mice using the MDA-MB-231 model of Kindlin-1 loss did not affect the final tumour volume (Fig. 23B). As it is difficult to establish tumours in mice using the MDA-MB-231 cell line in the CD1 nude mice, we chose to use SCID mice for our second primary tumour experiment using the MDA-MB-231 model. We also transplanted tumour fragments into the mice in order to speed up the process. SCID mice have a mutation that results in both T and B cell deficiency (271) whereas CD1 nude mice have depleted T cells but functional B cells (229). As SCID mice are more immunocompromised than CD1 mice, tumours established from human cell lines grow more easily in SCID mice compared to CD1 nude mice. However, we did not expect a difference in the growth trends with Kindlin-1 loss between the two mouse strains. There is no evidence to

date that Kindlin-1 effects B cell populations and experiments in CD1 and SCID mice would need to be repeated with identical conditions to test for this. It is possible that we saw a difference in tumour growths in the SCID mice compared to the CD1 mice because tumour fragments were transplanted into the SCID mice. Tumour fragments may react differently with loss of Kindlin-1 as they are established tumours and would have bypassed the tumour initiation phase that cells would undergo. This may mean that tumours are able to cope better with Kindlin-1 loss and therefore, we do not see a difference in the final tumour volumes in the SCID mice. The data from the CD-1 mice demonstrates a significant effect on the growth rate in the early stages of the experiment (at day 7) with knockdown of Kindlin-1, which conforms with this hypothesis. As we have shown that tumour initiation is affected with loss of Kindlin-1-integrin binding in the Met-1 model, taken together, this data provides more evidence to suggest that Kindlin-1 is important for the initial establishment of the tumour.

In order to establish whether the significant difference in tumour volumes in the CD1 nude mice is reproducible, the experiment needs to be repeated using the CD1 nude mice. In addition, generating a Kindlin-1 knockout MDA-MB-231 cell line would be useful to directly compare loss of Kindlin-1 in this model with the Met-1 model. Knockdown of Kindlin-1 using shRNA is not a clean model of Kindlin-1 loss, some Kindlin-1 expression will be present in the cell and this may account for smaller changes in tumour growth.

3.9.4 Summary and Future Work

In summary, our data strongly suggest that Kindlin-1 has a role in stem cell signalling, however, we have shown that Kindlin-1 does not act through the Notch pathway in a murine model of breast cancer and further experiments need to be carried out to investigate other pathways that Kindlin-1 may regulate. Further work to comprehensively analyse stem cell markers and cell surface markers combined with

in vivo transplantation paradigms or *in vivo* lineage tracing experiments would provide a greater understanding into the role of Kindlin-1 in stem cell regulation.

We have shown that Kindlin-1 regulates primary breast tumour formation and is important for the initial stages of tumour establishment in a mouse and human model. We have also demonstrated a novel role for Kindlin-1 in the regulation of T cells. We show that Kindlin-1 inhibits IL-6 production, which promotes differentiation of naïve CD4+ T cells into Tregs and results in tumour development due to suppression of the anti-tumour response of the immune system. For future studies, it would be valuable to further investigate the regulation of IL-6 by Kindlin-1 using an IL-6 inhibitor *in vivo* and by analysing effector T cell populations.

Chapter 4 Adaptor Functions of Kindlin-1

It has already been shown that all three of the Kindlin isoforms bind to ILK. Kindlin-3 was found to bind to ILK with the least affinity whilst Kindlin-2 was found to bind with the strongest affinity (160). ILK is an important regulator of integrin-mediated processes and its main function is as an adaptor protein partnering with the LIM domain protein, PINCH and the calponin homology domain protein, parvin to form the IPP complex, which is imperative for its function (162,272–276). ILK also binds to other FA proteins either directly or indirectly through either PINCH or parvin in order to mediate integrin-signalling and function as a scaffold protein at FAs (161,277–279). ILK has therefore been linked to numerous cellular processes, which include adhesion, differentiation, survival, migration and polarity, proliferation and invasion (as reviewed in (280)).

Research has revealed that binding of Kindlin-2 to the IPP complex supports integrin activation (160). Binding of ILK to Kindlin also drives subcellular localisation of the Kindlins, however, work has focussed mainly on Kindlin-2 and Kindlin-3 and less is known about the role of Kindlin-1-ILK binding (160,281). Therefore, we wanted to establish if Kindlin-1 interacts with ILK similarly to Kindlin-2 at focal adhesions in a breast cancer and squamous cell carcinoma model. Furthermore, the importance of the role of Kindlin as an adaptor protein is clearly highlighted through its interaction with ILK. Therefore, we wanted to understand how the interaction of Kindlin-1 with other proteins may be significant and identify novel binding partners.

Specific aims of this chapter include:

1. Use immunofluorescence to identify if the subcellular localisation of ILK is dependent on Kindlin-1

2. Identify novel binding partners of Kindlin-1 in breast cancer cell models

4.1 ILK localises to the focal adhesions in the presence and absence of Kindlin-1 in two different cancer models

Because Kindlin-1 is known to bind to ILK in CHO cells (160), we first wanted to confirm that ILK binds Kindlin-1 in the Met-1 and SCC models. Models of Kindlin-1 loss were previously generated in Met-1 (185) and SCC (210) cells. Similarly to generation of the Met-1 model previously described in chapter 3, WT-Kindlin-1 was re-expressed into SCC Kin1-Null cells to generate SCC Kindlin-1 WT cells (Kin1-WT) (210). Additionally, a mutant Kindlin-1, in which glutamine 611 and tryptophan 612 was mutated to alanine, was re-expressed into SCC Kin-1 Null cells to generate SCC Kindlin-1 AA cells (Kin1-AA) (210). As explained in chapter 3, tryptophan 612 is critical for integrin binding and its mutation to alanine prevents Kindlin-1 binding to β-integrin tails (145). The Kin-1 AA cell line enables the role of Kindlin-1 and integrin activation to be investigated in both cell lines. Immunoprecipitation using a Kindlin-1-specific antibody was able to coimmunoprecipitate ILK confirming that Kindlin-1 binds ILK in both the Met-1 and SCC models (Fig. 30). The loss of integrin binding (Kin1-AA) did not impact Kindlin-ILK binding in SCC cells (Fig. 30B). As Kindlin-1 binds ILK in both of these models, we next wanted to establish if loss of Kindlin-1 would impact the recruitment of ILK to FAs. In order to visualise FAs, we stained for the well-characterised focal adhesion protein, FAK (282). In the absence of Kindlin-1, ILK is still recruited to FAs and colocalises with FAK in the Met-1 (Fig. 31) and SCC (Fig. 32) models. The loss of integrin binding (Kin1-AA) did not impact ILK localisation to FAs in the SCC cells (Fig. 32). It has previously been shown that the Kindlin-2-ILK interaction is necessary for FA localisation and so, as these cells also express Kindlin-2, it is possible that Kindlin-2 can compensate for Kindlin-1 loss in these models and recruit ILK to FAs (281). It was not possible to stain for Kindlin-1 by immunofluorescence (IF) as we found both the Abcam Kindlin-1 antibody and the Movarian-biotech Kindlin-1 antibody ineffective.

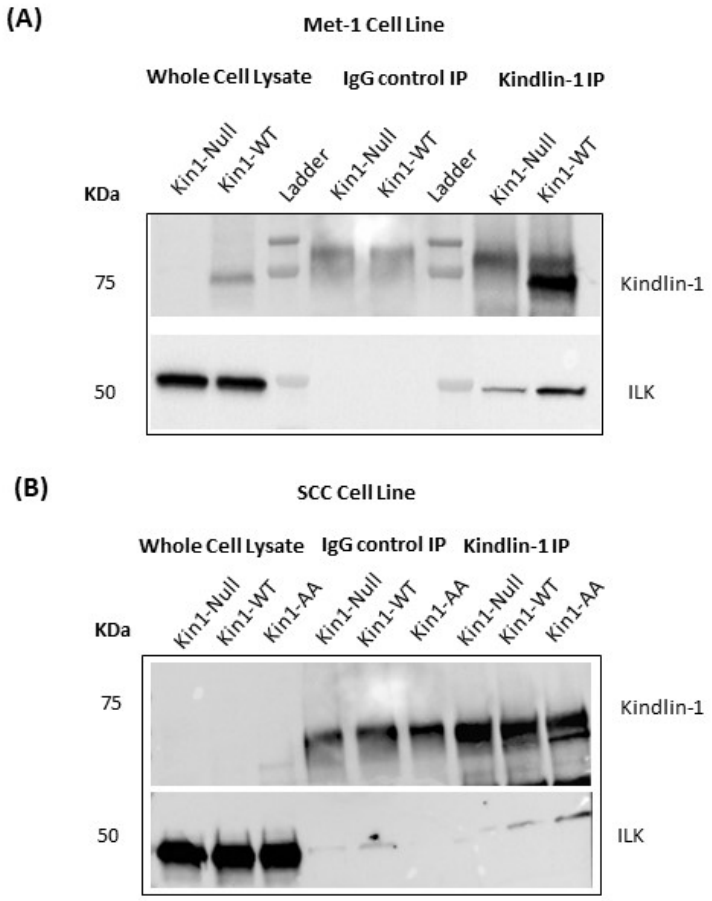


Figure 30. Kindlin-1 binds to ILK in two different cancer models. Western blot analysis demonstrating that immunoprecipitation using a Kindlin-1-specific antibody was able to co-immunoprecipitate ILK in (A) Met-1 and (B) SCC whole cell lysates.

4.2 Identification of novel Kindlin-1 binding partners

FAs provide a mechanical link between the ECM and the actin cytoskeleton through the interaction of the transmembrane proteins, integrins, with the ECM and with intracellular multi-protein assemblies that connect to the actin cytoskeleton (283). FAs are responsible for directing signalling from many proteins and inducing biochemical and mechanical outputs. Over sixty focal adhesion proteins have been discovered in vertebrates, which play catalytic and/or adaptor roles at focal adhesions (283). As very few Kindlin-1-focal adhesion protein binding partners have been determined (Fig. 7), immunoprecipitation with a Kindlin-1-specific antibody followed by mass spectrometry analysis was performed using the mouse Met-1

model and the human MDA-MB-231 cell model in order to identify novel Kindlin-1 binding partners.

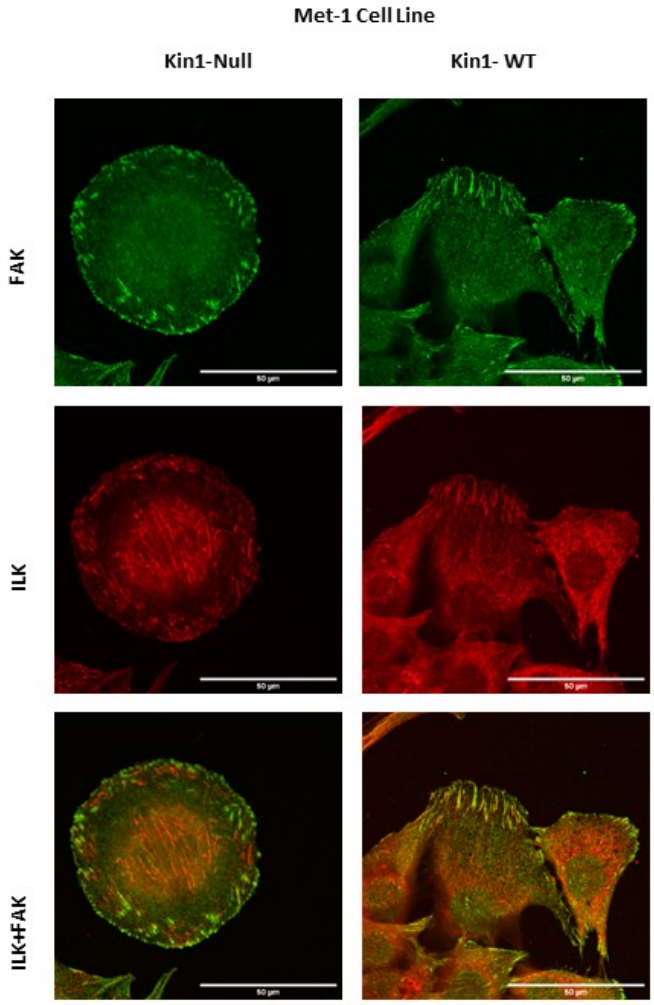


Figure 31. ILK is still able to localise at focal adhesions with Kindlin-1 loss in Met-1 cells. Confocal images of immunofluorescence staining of DAPI (blue), ILK (red) and FAK (green) in Met-1 cells. Scale bar: 50µm. Magnification 60x.

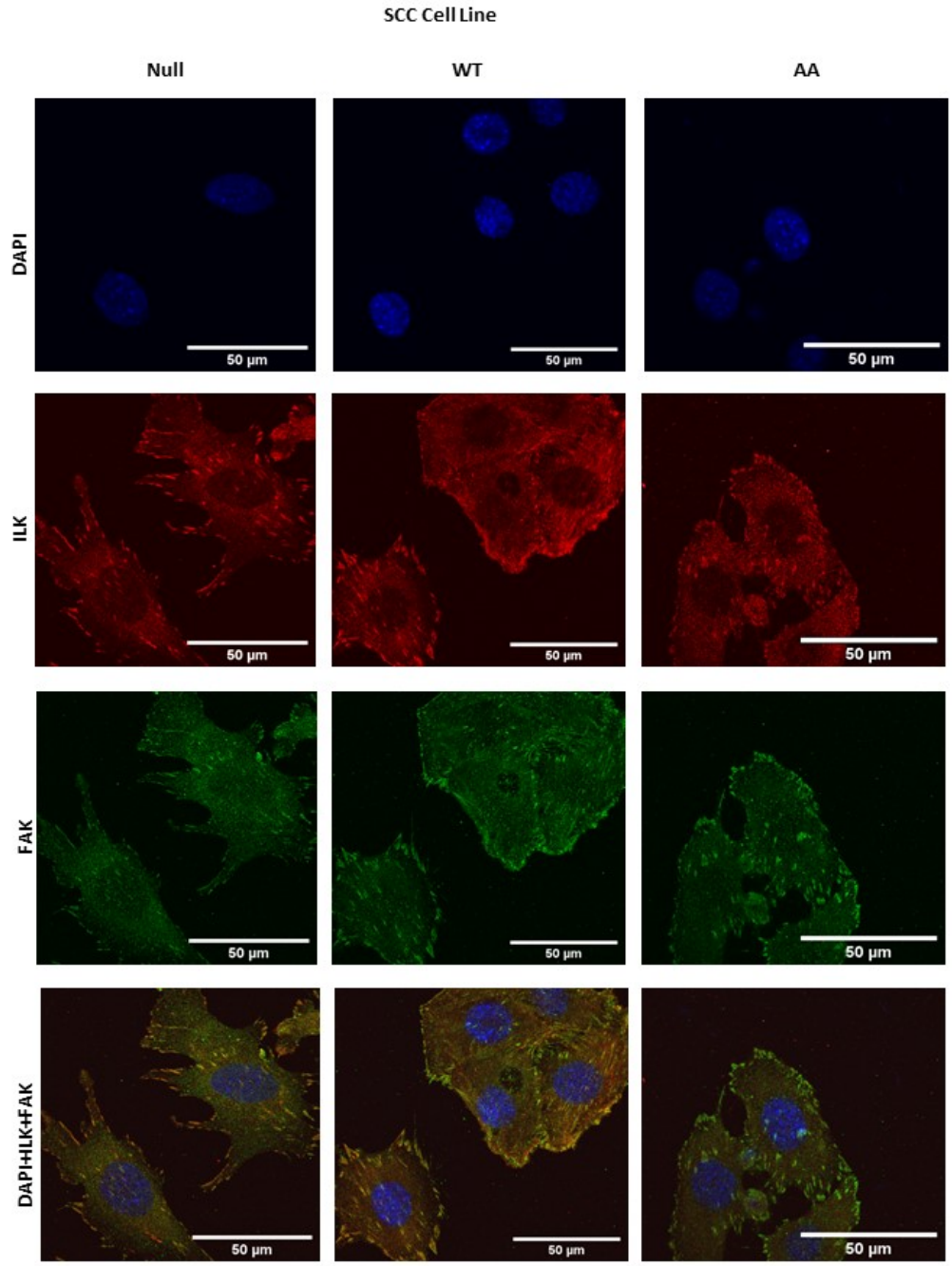


Figure 32. ILK is still able to localise at focal adhesions with Kindlin-1 loss in SCC cells. Confocal images of immunofluorescence staining of DAPI (blue), ILK (red) and FAK (green) in SCC cells. Scale bar: 50µm. Magnification 60x.

4.2.1 Kindlin-1 binding partners in the Met-1 model

Hierarchical clustering of the immunoprecipitated samples using Met-1 whole cell lysates demonstrated that samples from all of the three cell lines (Met-1 Kin1-Null, Kin1-WT and Kin1-AA) immunoprecipitated using the control IgG antibody, cluster together (Fig. 33). Samples using Met-1 Kin1-Null whole cell lysates immunoprecipitated with the Kindlin-1 antibody cluster alongside the control IgG samples demonstrating that immunoprecipitation with the Kindlin-1 antibody has enriched for Kindlin-1 associated proteins in the Met-1 Kin1-WT and Kin1-AA whole cell lysates (Fig. 33).

Principal component analysis shows clusters of samples based on their similarity and demonstrates that samples from all cell lines immunoprecipitated with the control IgG antibody cluster together (Fig. 34A). Samples from the Kin1-WT and Kin1-AA cell lines that were immunoprecipitated with the Kindlin-1 antibody cluster together away from the Kin1-Null samples (Fig. 34B). Volcano plots show a clear difference in protein abundance between the Kin1-Null and Kin1-WT cell lines (Fig. 35A) and between the Kin1-Null and Kin1-AA cell lines (Fig. 35B). Samples from the Met-1 Kin1-WT and Kin1-AA cell lysates immunoprecipitated with the Kindlin-1 antibody cluster together and the heat map shows that protein abundance is similar between the samples (Fig. 33). This was reaffirmed by the volcano plot, which shows that there is no significant difference in proteins binding to Kindlin-1 between the Kin1-WT and Kin1-AA cell lines (Fig. 35C).

We decided to analyse the difference between the Kin1-Null and Kin1-WT cell lines only. A Students t-test identified that 70 proteins significantly bound to Kindlin-1 in Kin1-WT cells with an enrichment factor of 1.5-fold or greater (Fig. 36A), with 18 proteins enriched greater than 2-fold (Table 11, Fig. 36B). As predicted, ILK was found amongst the list of genes binding to Kin1-WT, however, the two other members of the ILK complex (PINCH and parvin) were not found in the data set (Fig. 36). Network

analysis of the 70 proteins with an enrichment factor of 1.5-fold identified several hubs linked to the T-complex 1 (TCP1) protein-folding complex, components of the 26S proteasome and proteins involved in the cell cycle and microtubule-based processes (Fig. 36A, 37C). The majority of these proteins have binding or catalytic activities (Fig. 37A) and are involved in cellular and metabolic processes (Fig. 37B).

It has previously been shown that Kindlin-1 is involved in the cell cycle by regulating mitotic spindle assembly through phosphorylation of Plk1 (168). Kindlin-1 has also been shown to regulate microtubule function to enable mitosis (284), and four of the proteins identified through the proteomic analysis are involved in microtubule-based processes. These include: Tubulin Beta Class V (Tubb6), Valosin-containing protein (Vcp), Tubulin Beta 4B Class IVb (Tubb4b) and Dynein Cytoplasmic 1 Heavy Chain 1 (Dync1h1). Interestingly, TUBB4B is known to regulate Plk1 at G2/M transition. Network analysis of the proteins involved in the cell cycle and microtubule-based processes identified in the data set may shed insight into how Kindlin-1 may be regulating cell cycle progression (Fig. 37D). Of significance, are the proteins of the mini-chromosome maintenance (MCM) family: Mcm6 and Mcm5. The MCM family of proteins are essential for initiating genome replication in eukaryotes and are key players of genome stability as they have been associated with DNA damage response, transcription and chromatin structure (285). Their expression is stable throughout the cell cycle and therefore, they are considered a sensitive marker of proliferation and high levels of these proteins are associated with tumour or precancerous cells (286).

Other hits of interest include the eukaryotic translation elongation factor, eEF1A1, which is highly expressed in breast tumours and modulates the cytoskeleton (287) and proteins that are components of the 26S proteasome (Fig. 36), which is known to be involved in multiple cell processes such as the cell cycle and apoptosis (288).

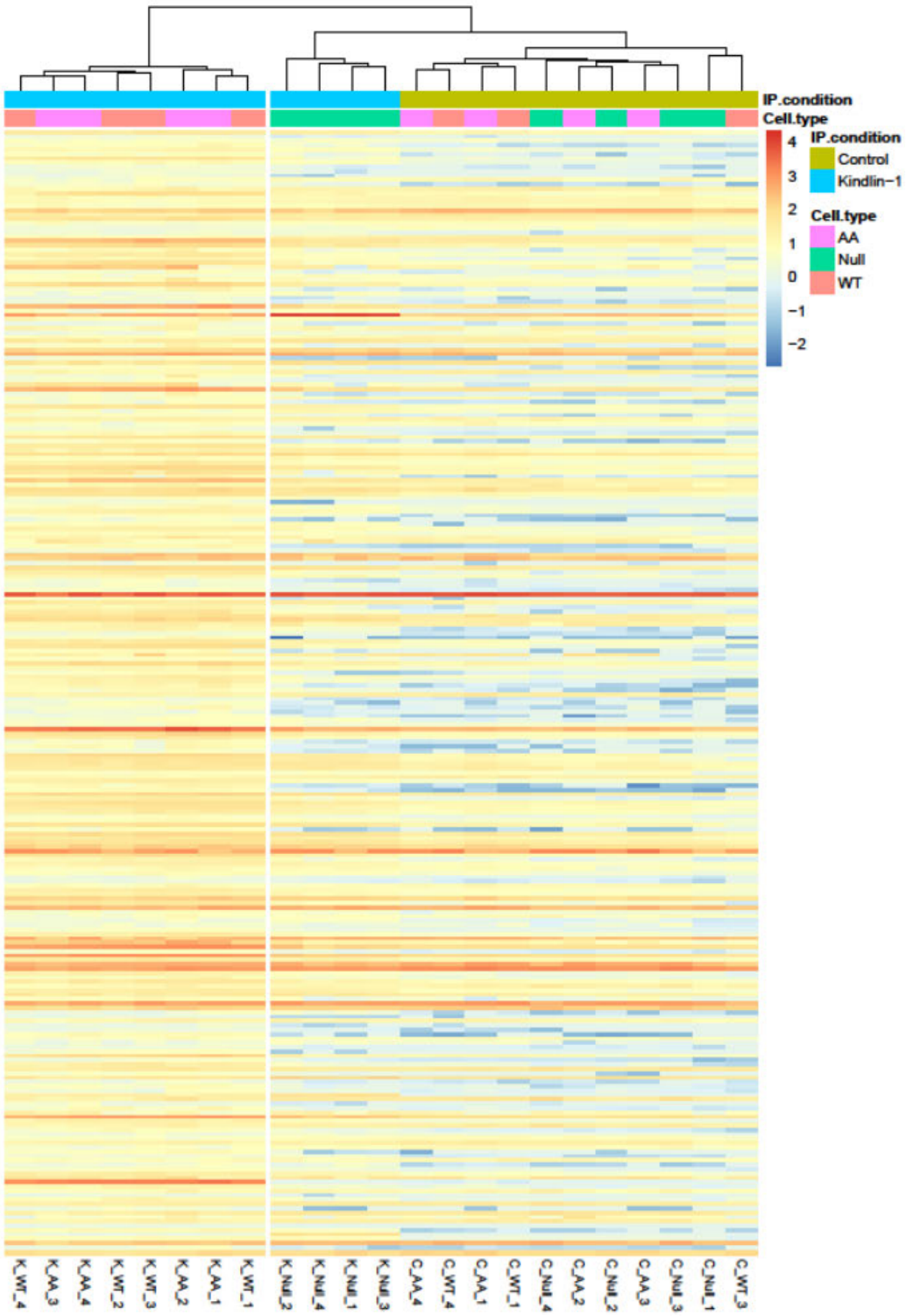


Figure 33. Heat map with hierarchical clustering of label-free quantification (LFQ) values of proteins identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) that interact with Kindlin-1 from Met-1 whole cell lysates. "K" and "C" correspond to immunoprecipitation using either the Kindlin-1 or control IgG antibody and the numbers correspond to the technical replicates of each of the samples. The colours correspond to protein-relative abundance, using Z score normalised values (Kindlin-1 bait abundance is set to 1). On the bait-normalised scale, red and shades of red correspond to abundant interactions between Kindlin-1 and the hits, while white to blue colour indicates barely detectable or non-detectable interaction.

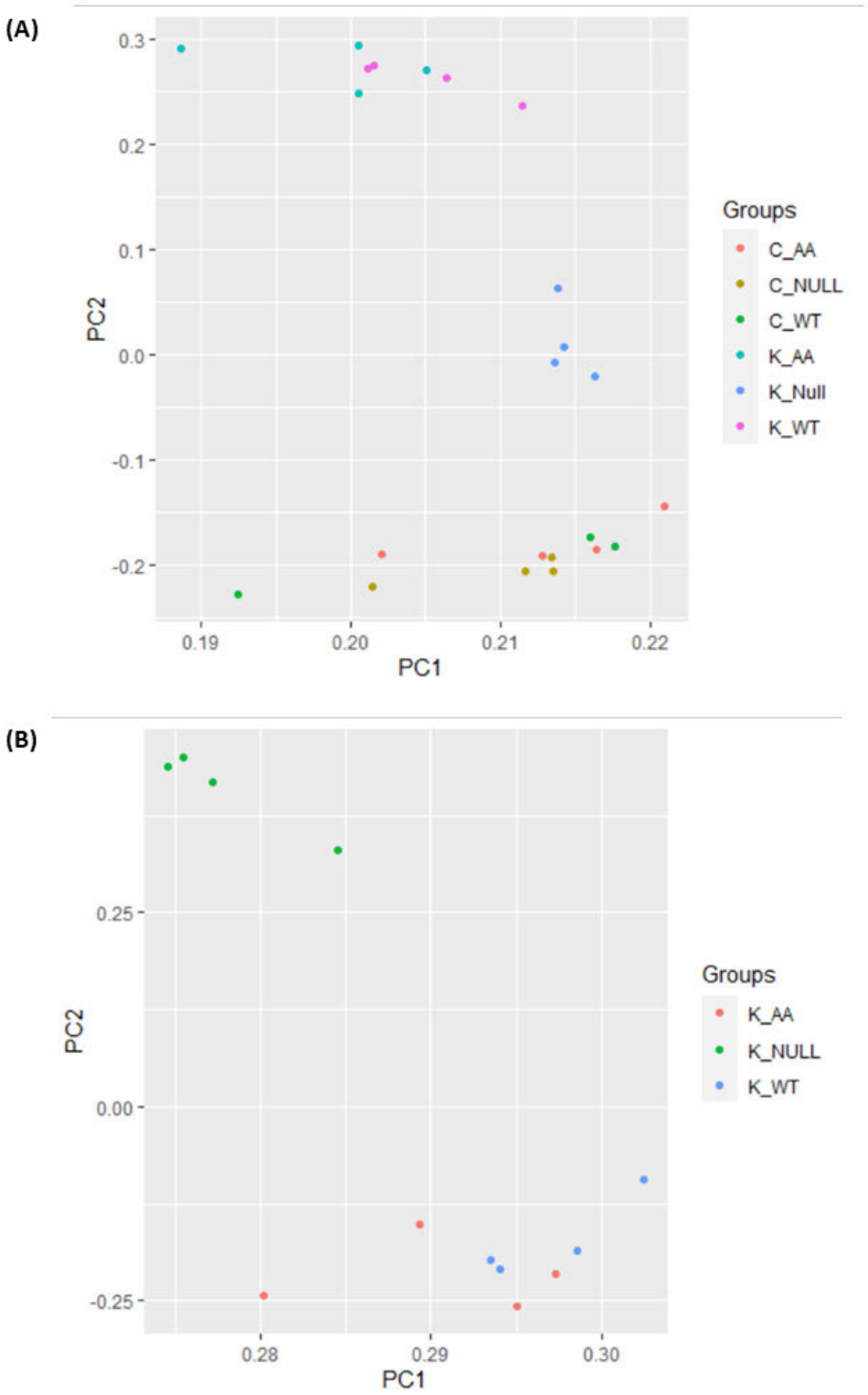


Figure 34. Principal component analysis (PCA) of samples from liquid chromatography-tandem mass spectrometry (LC-MS/MS). Samples are colour coded by cell line and condition. "K" and "C" correspond to immunoprecipitation using either the Kindlin-1 or control IgG antibody. (A) PCA of samples immunoprecipitated using either the Kindlin-1 or control IgG antibody (B) PCA of samples immunoprecipitated using the Kindlin-1 antibody.

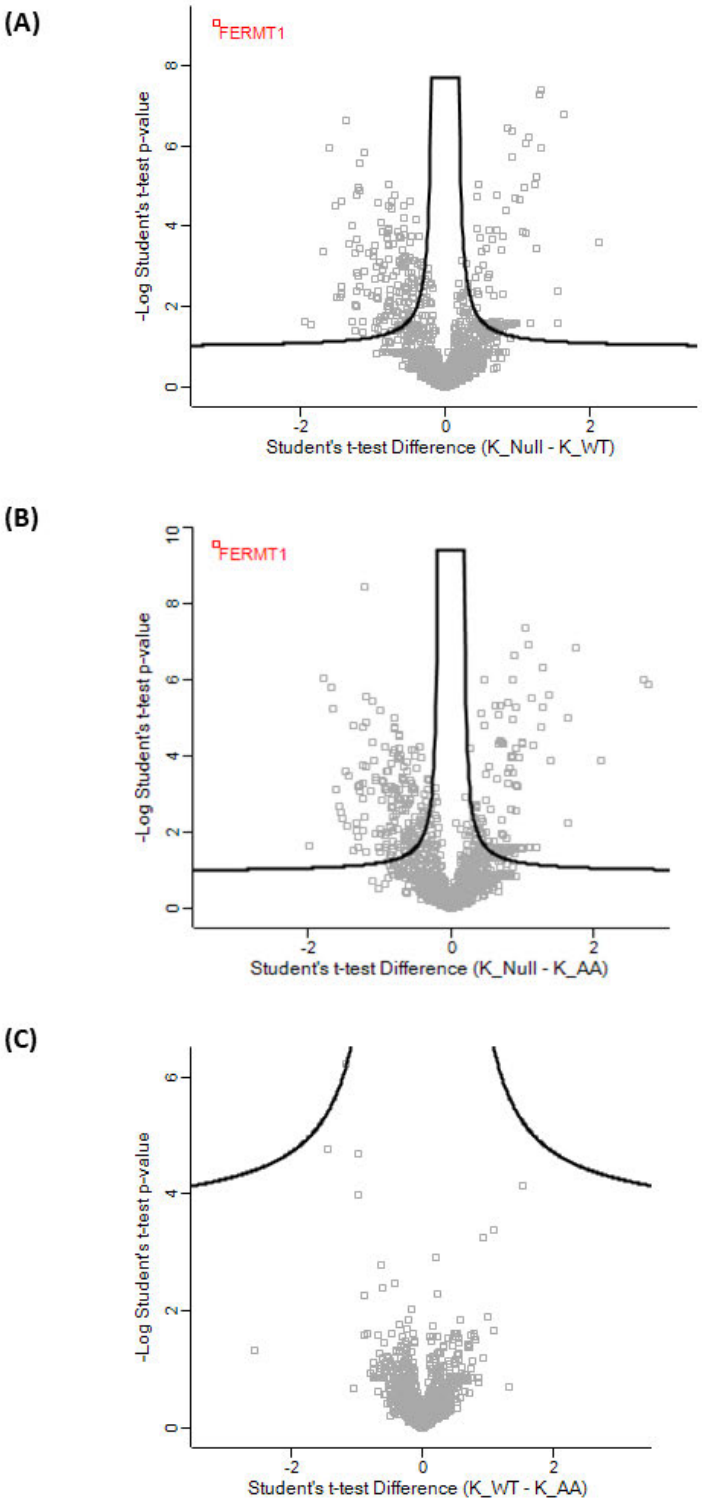


Figure 35. Volcano plots of the mass spectrometry data from the Met-1 model. Volcano plots of the mass spectrometry data demonstrate the magnitude and significance of the cellular proteins interacting with (A) Kindlin-1 WT (K_WT) and (B) Kindlin-1 AA (K_AA) compared to the negative control Kindlin-1 Null (K_Null). No difference was found between proteins interacting with K_WT compared to K_AA (C). The x-axis is the fold-change value (Kindlin-1 WT or Kindlin-1 AA/ Kindlin-1 Null) and the y-axis is –log10 p-value showing statistical significance.

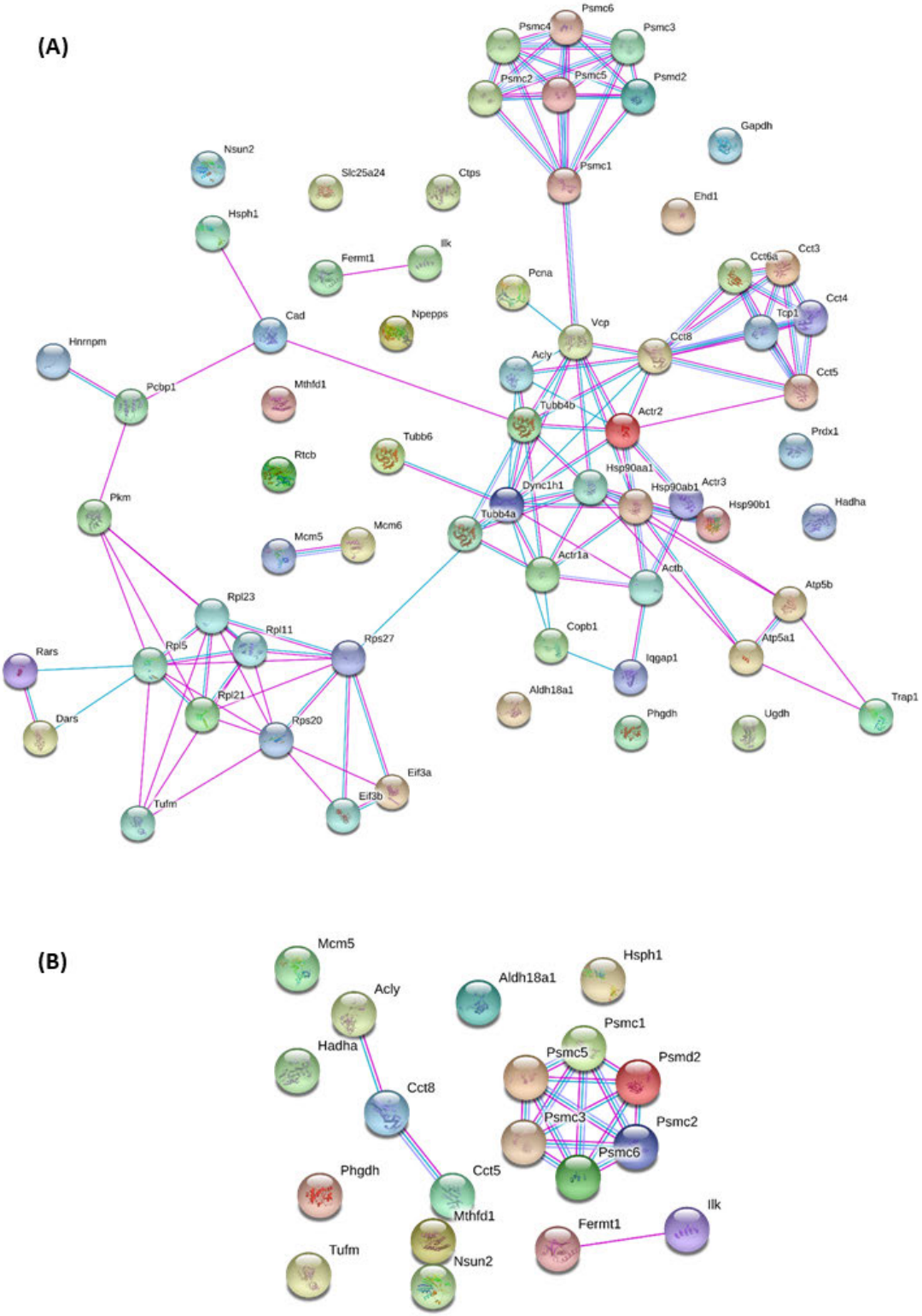


Figure 36. Met-1 Kindlin-1 WT interactome. A Students t-test ($p<0.05$) identified that 70 proteins (A) significantly bound to Kindlin-1 in WT cells with an enrichment factor of 1.5-fold or greater and 18 (B) were enriched greater than 2-fold. Networks were generated using STRING.

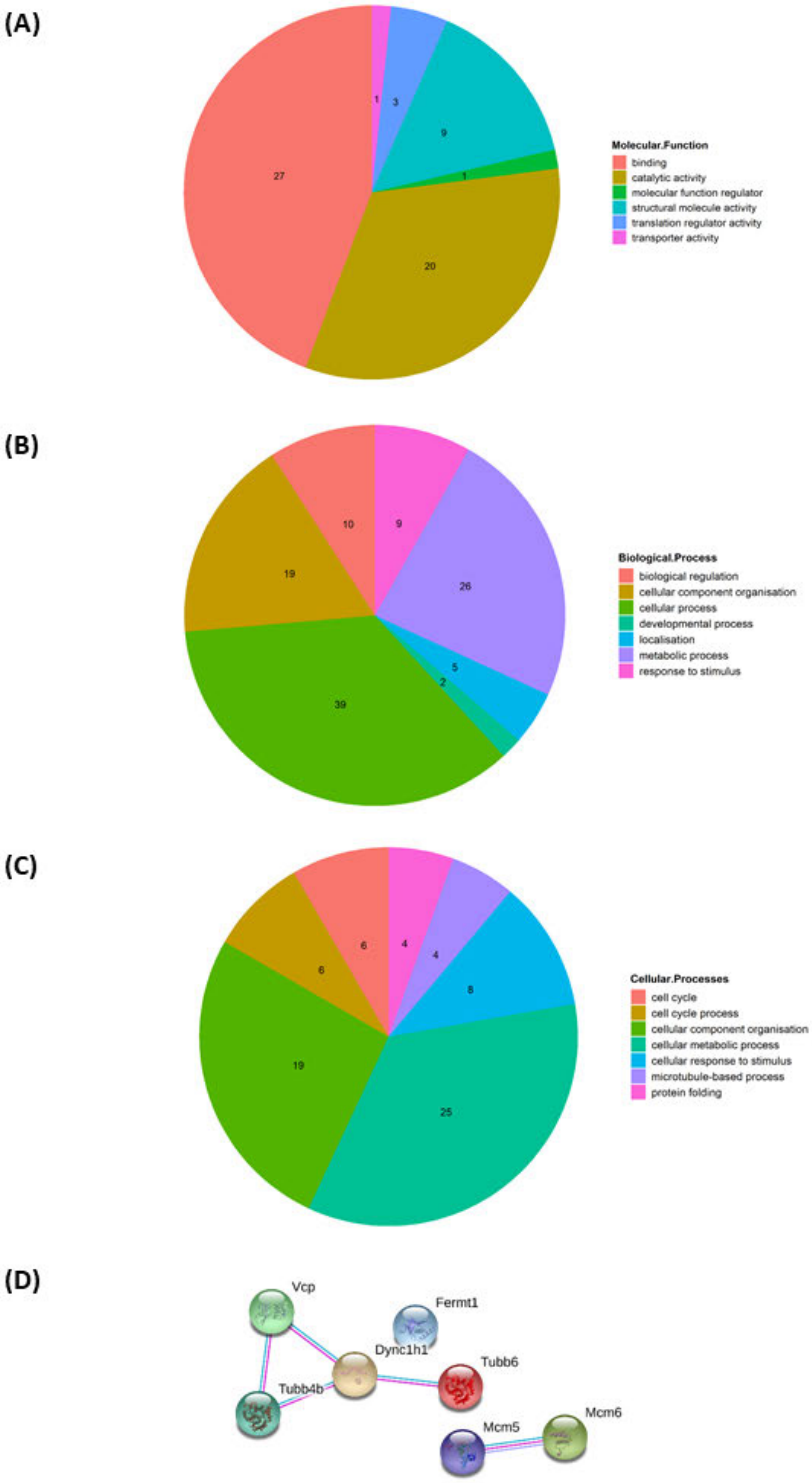


Figure 37. Gene Ontology analysis of Kindlin-1 binding proteins. Pie charts show the (A) Molecular Functions, (B) Biological Processes and (C) Cellular Processes of the 70 proteins that significantly bound ($p<0.05$) to Kindlin-1 in WT cells with an enrichment factor of 1.5-fold or greater. (D) Network analysis of the proteins involved in the cell cycle and microtubule-based processes using STRING.

Table 11. Functions of the 18 proteins identified by a Students t-test that significantly bound to Kindlin-1 in WT cells with an enrichment factor greater than 2-fold.

Gene Name	Function
PSMC2	Proteasome 26S subunit, ATPase 2 (PSMC2) is an ATPase subunit of the 26S proteasome, which is a multiprotein complex that functions to degrade ubiquitinated proteins in an ATP-dependent manner. High expression of proteasome subunits has been found in different cancers including breast cancer and this correlates with the necessity of this complex for proliferation of tumour cells in tissues (289).
NSUN2	NOP2/Sun RNA Methyltransferase 2 (NSUN2) is a tRNA methyltransferase that methylates mRNAs. It regulates the translation or turonover of mRNAs that are involved in processes such as cell proliferation, oxidative stress and replicative senescence (290–294).
MTHFD1	Methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1 (MTHFD1) is a metabolic enzyme that catalyses tetrahydrofolate into substrates for de novo purine and pyrimidine synthesis (295). MTHFD1 is upregulated in several cancers and has been associated with poor disease outcomes in breast cancer (296,297).
PSMC1	Proteasome 26S subunit, ATPase 1 (PSMC1) is an ATPase subunit of the 26S proteasome (see above description for PSMC2).
HADHA	Alpha subunit of the mitochondrial enzyme, hydroxyacyl-CoA dehydrogenase trifunctional multi-enzyme (HADHA), which catalyses the last three reactions of mitochondrial beta-oxidation of long chain fatty acids (298). HADHA is downregulated in clear cell renal carcinoma and when overexpressed, has been shown to inhibit cancer growth by disrupting lipid metabolism (299).
ACLY	ATP-citrate lyase (ACLY) is an enzyme that cleaves citrate and coenzyme A to form oxaloacetate and acetyl-CoA. These molecules are essential to sustain pathways that support cancer cell growth (as reviewed in (300).
PSMC3	Proteasome 26S subunit, ATPase 3 (PSMC3) is an ATPase subunit of the 26S proteasome (see above description for PSMC2).
PSMC6	Proteasome 26S subunit, ATPase 6 (PSMC6) is an ATPase subunit of the 26S proteasome (see above description for PSMC2).
PHGDH	Phosphoglycerate dehydrogenase (PHGDH) is an enzyme involved in the initial step of the serine biosynthetic pathway. PHGDH is highly expressed in cancers including breast cancer (301).
PSMC5	Proteasome 26S subunit, ATPase 5 (PSMC5) is an ATPase subunit of the 26S proteasome (see above description for PSMC2).
TUFM	Mitochondrial Tu translation elongation factor (TUFM) is a protein present in the mitochondria that that is involved in mitochondrial protein translation. Upregulation of TUFM has been reported in several cancers (302–306).
PSMD2	Proteasome 26S subunit, non-ATPase 2 (PSMD2) is a non-ATPase subunit of the 26S proteasome (see above description for PSMC2).

HSPH1	Heat shock protein family H member 1 (HSPH1) is a member of the heat shock protein 70 family. Heat shock proteins (HSPs) are chaperones involved in refolding of misfolded proteins and regulate other important cellular processes including secretion, transportation, translocation and protein degradation. They also regulate transcription factors. HSPs are overexpressed in a range of different cancers and many of the proteins they interact with are well known oncoproteins. (as reviewed in (307))
ALDH18A1	Aldehyde dehydrogenase 18 family member A1 (ALDH18A1) is a member of the aldehyde dehydrogenase family, which are a family of enzymes that detoxify intracellular aldehydes. High ALDH1 expression correlates with poor prognosis in breast cancer and has been linked to metastasis (51,308). In breast cancer, cells with high expression of members of the ALDH family have cancer stem cell properties (25,308).
CCT8	Chaperonin containing t-complex polypeptide 1 subunit 8 (CCT8) is a subunit of the chaperonin, chaperonin containing t-complex polypeptide 1 (CCT). Substrates for CCT are involved in protein folding and the subunits of the complex have been linked to different cancer types including breast cancer (309).
ILK	ILK is a FA protein that is required for effective integrin signalling and essential for development (161,310). It bind with PINCH and Parvin to form a mutiprotein complex that is necessary for its function (162,272–276). Its functions include: adhesion, differentiation, survival, migration and polarity, proliferation and invasion (as reviewed in (280).
MCM5	Minichromosome maintenance complex component 5 (MCM5) is a member of the minichromosome (MCM) family. The MCM family of proteins are involved in genome replication in eukaryotes and have roles in the DNA damage response, transcription and chromatin structure (285). Their expression is a marker of proliferation and therefore, their expression has been linked to cancer (286).
CCT5	Chaperonin containing t-complex polypeptide 1 subunit 5 (CCT5) is a subunit of the chaperonin, chaperonin containing t-complex polypeptide 1 (CCT). See above description for CCT8.

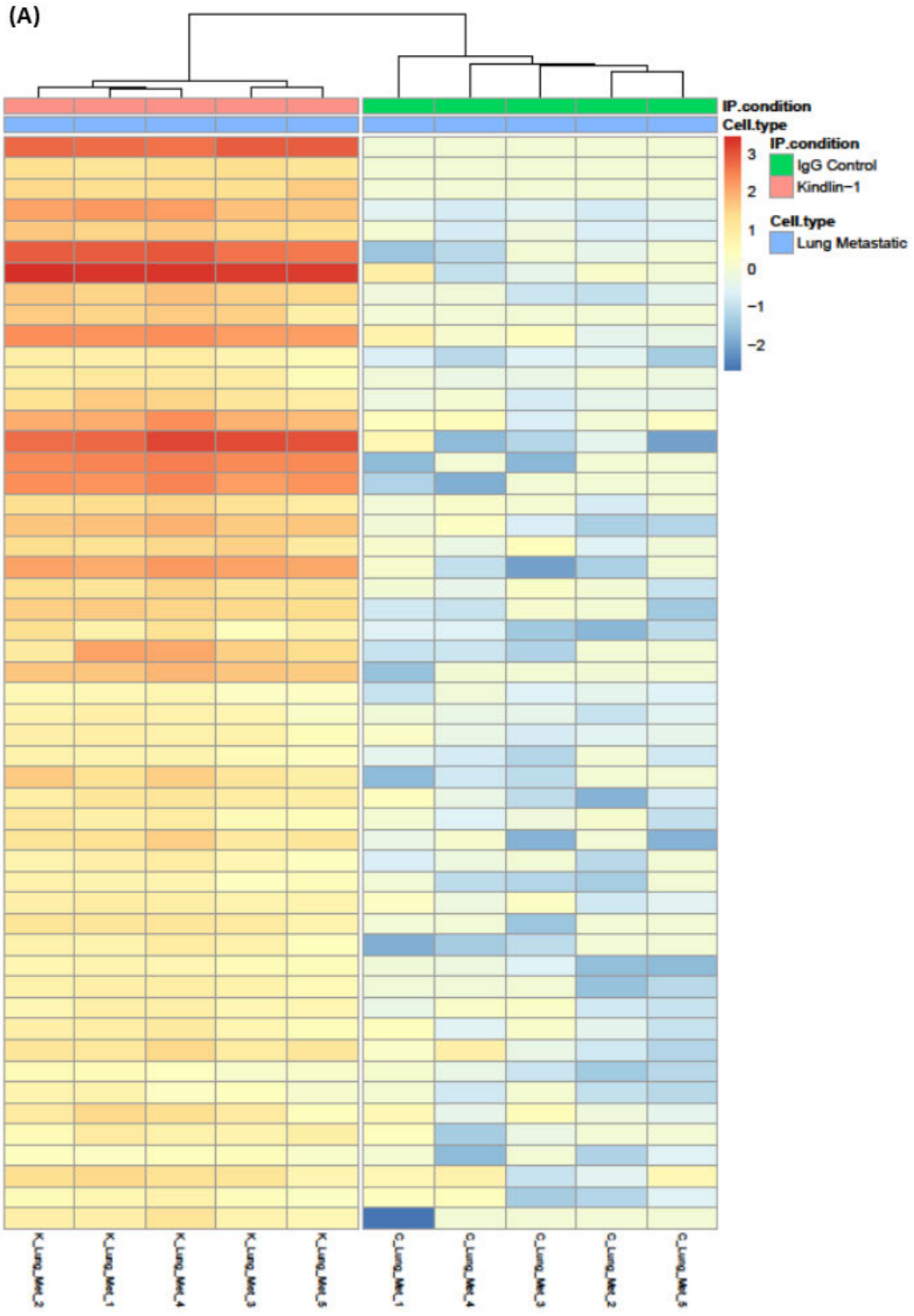
We chose to follow up VCP and Dync1h1 from the Kindlin-1 binding hits in the Met-1 model and verify these hits by co-IP. VCP appeared to be a contaminant in the data set based on co-IP experiments that were carried out (not shown). Western blot analysis showed that VCP was present in both Met-1 Kin1-Null and Kin1-WT cells immunoprecipitated with Kindlin-1. The CRAPome database (a contaminant repository for affinity purification coupled with mass spectrometry) also shows VCP as a contaminant in multiple affinity purification experiments that have been coupled with mass spectrometry (311). Although an initial Dync1h1 co-IP experiment showed that Kindlin-1 binds to Dync1h1 in Met-1 Kin1-WT and Kin1-AA cells, the data was

very variable upon repeat. There was not enough time to further optimise the conditions of the co-IP. For future experiments, different beads and different IP conditions could be tested.

4.2.2 Kindlin-1 binding partners in the MDA-MB-231 model

To look for common Kindlin-1 binding partners in different breast cancer models, immunoprecipitation with a Kindlin-1-specific antibody followed by mass spectrometry analysis was performed with the human MDA-MB-231 cell line. The experiment was carried out using a lung metastatic variant of the human MDA-MB-231 cell line (MDA-MB-231-LM), alongside its parental counterpart (207). Hierarchical clustering of MDA-MB-231 whole cell lysates immunoprecipitated with the Kindlin-1-specific antibody cluster away from those immunoprecipitated with the control IgG antibody in both cell lines (Fig. 38A-B). Principal component analysis demonstrates that samples from cell lines immunoprecipitated with the control IgG antibody cluster together and away from the samples immunoprecipitated with the Kindlin-1 antibody (Fig. 39).

More proteins were significantly differentially bound to Kindlin-1 with an enrichment factor of 2-fold in the lung metastatic variant (Fig. 40A,41B) compared to its parental counterpart (Fig. 40B,41B) when normalised against the IgG controls (51 compared to 35, respectively). Table 12 shows a list of genes only found in the lung metastatic cell line or the parental cell line. Similarly, to the mouse breast cancer model, gene ontology analysis showed that the majority of the proteins bound to Kindlin-1 in the parental and lung metastatic MDA-MB-231 cell lines have binding or catalytic activities (Fig. 42A,43A) and are involved in cellular and metabolic processes (Fig. 42B,43B). Some of these proteins are also involved in the cell cycle and cell cycle processes as found in the Met-1 data set (Fig. 42C,43C).


(A)
IP.condition
Cell.type
IP.condition
IgG Control
Kindlin−1
Cell.type
Lung Metastatic
3
2
1
0
−1
−2
K_Lung_Met_2
K_Lung_Met_1
K_Lung_Met_4
K_Lung_Met_3
K_Lung_Met_5
C_Lung_Met_1
C_Lung_Met_4
C_Lung_Met_3
C_Lung_Met_2
C_Lung_Met_5

(B)

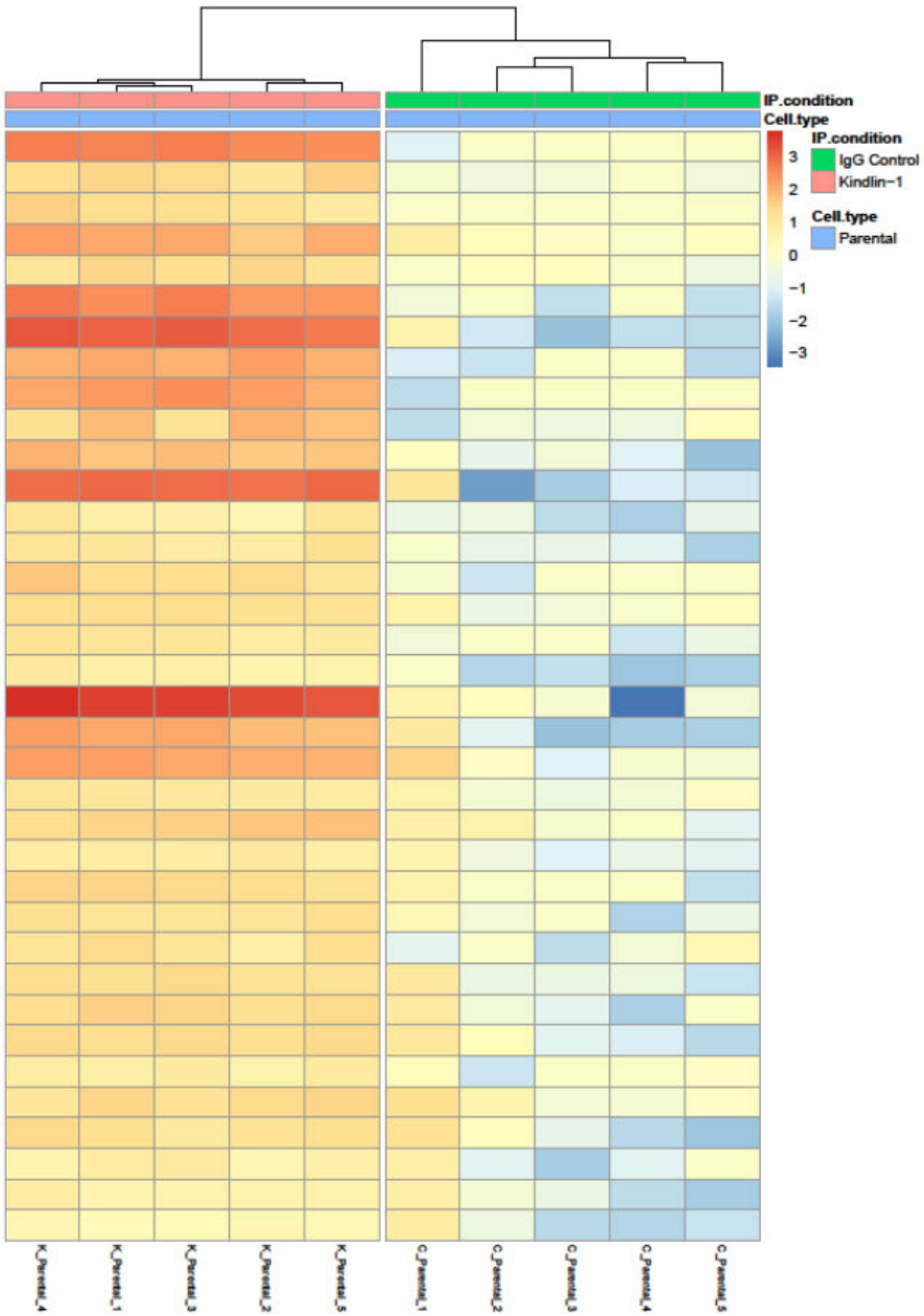


Figure 38. Heat maps with hierarchical clustering of label-free quantification (LFQ) values of proteins identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) that interact with Kindlin-1 from MDA-MB-231 whole cell lysates. Cellular proteins interacting with Kindlin-1 compared to a negative IgG control in (A) Lung metastatic and (B) Parental MDA-MB-231 whole cell lysates. **"**K" and "C" correspond to immunoprecipitation using either the Kindlin-1 or control IgG antibody and the numbers correspond to the technical replicates of each of the samples. The colours correspond to protein-relative abundance, using Z score normalised values (Kindlin-1 bait abundance is set to 1). On the bait-normalised scale, red and shades of red correspond to abundant interactions between Kindlin-1 and the hits, while white to blue colour indicates barely detectable or non-detectable interaction.

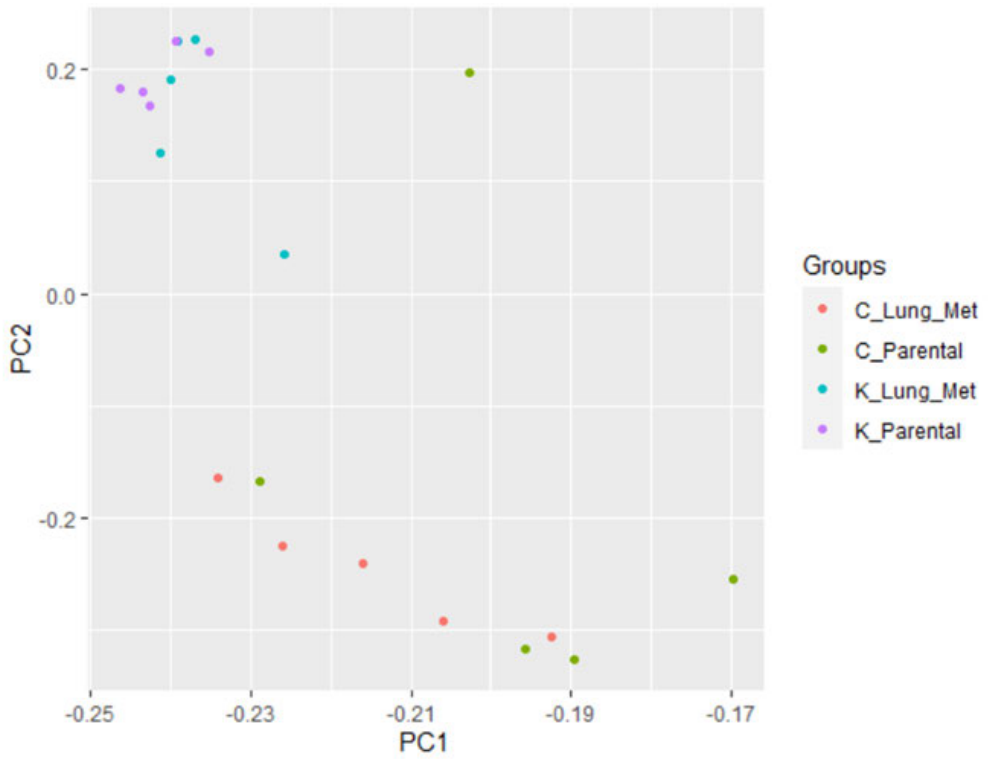


Figure 39. Principal component analysis (PCA) of samples from liquid chromatography-tandem mass spectrometry (LC-MS/MS). Samples are colour coded by cell line and condition. "K" and "C" correspond to immunoprecipitation using either the Kindlin-1 or control IgG antibody.

Network analysis showed that the interactome of the lung metastatic variant was very similar to that of the parental cell line (Fig. 44,45). An interesting family of proteins that were found in both these data sets were proteins from the 14-3-3 family, which mediate signal transduction by binding to phosphoserine-containing proteins and govern critical processes in cancer (312). β-catenin (*CTNNB1*) is also found in both cells lines and Kindlin-1 has previously been linked to increased canonical Wnt/β-catenin signalling (156). Notably, ILK was not present in the MDA-MB-231 proteomic data sets (Fig. 44,45). However, all three data sets contained proteins from the MCM family (Fig. 36,44,45) and similarly to the Met-1 data set, the lung metastatic variant data set contained proteins that form the 26S proteasome (Fig. 36,44). Although we saw proteins from the same family of proteins, there were no common Kindlin-1 interactors found between the three cell lines (Fig. 41A). Only one common binder was found between the Met-1 Kin1-WT and the MDA-MB-231 lung metastatic cell line (Fig 41A), which was the alpha subunit of the mitochondrial

enzyme, hydroxyacyl-CoA dehydrogenase trifunctional multi-enzyme (HADHA) (Fig. 36,44).

Table 12. Proteins significantly differentially bound to Kindlin-1 in only the Lung metastatic or Parental cell lines with an enrichment factor of 2-fold or above.

Lung metastatic only	Parental only
DNM2	UCHL3
HADHB	ACTR1A
XPO1	YWHAH
USO1	AP2A1
HUWE1	
TPM4	
PSMD12	
RUVBL2	
HADHA	
YWHAQ	
GBE1	
PSMC4	
HNRNPC;HNRNPCL4;HNRNPCL1;HNRNPCL3;HNRNPCL2	
NAA15	
RUVBL1	
NCAPG	
VPS35	
TUBB6	
KIF5B	
TMF1	

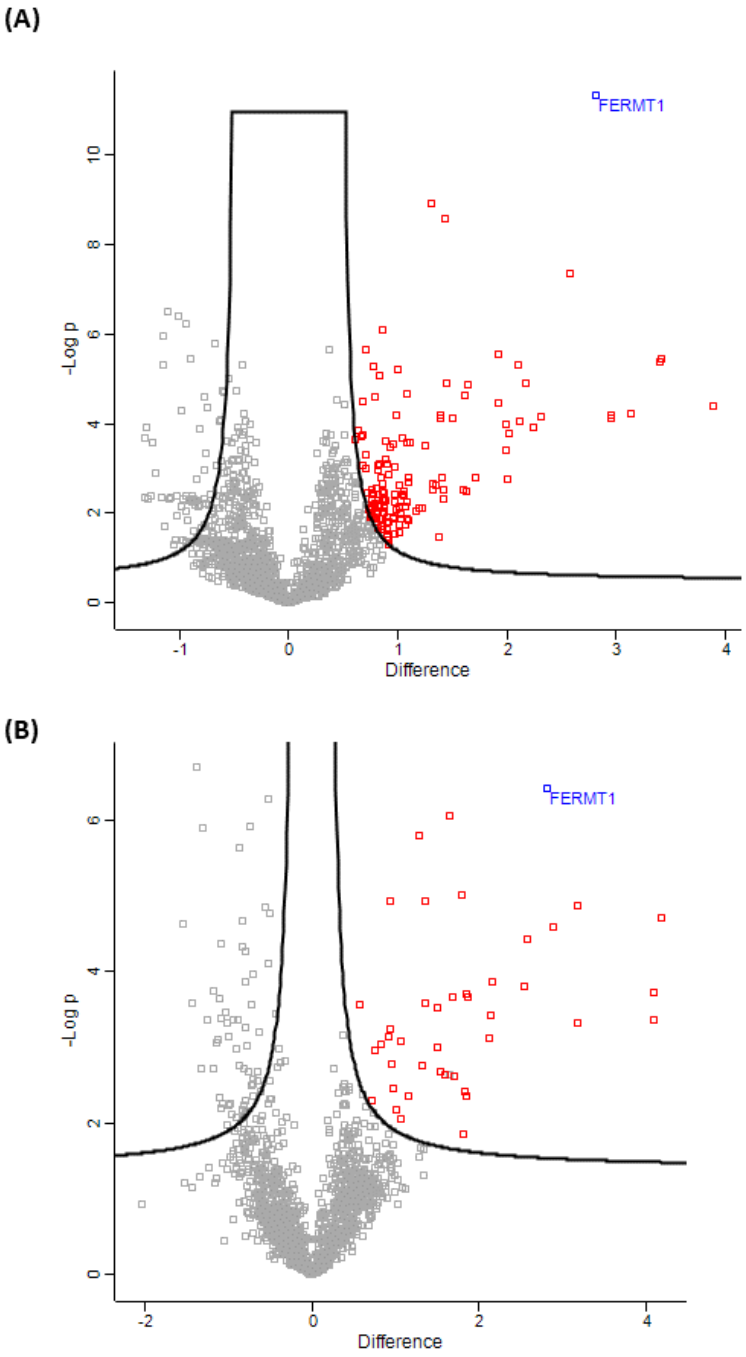


Figure 40. Volcano plots of the mass spectrometry data from the MDA-MB-231 cell lines. Volcano plots of the mass spectrometry data demonstrate the magnitude and significance of the cellular proteins interacting with Kindlin-1 compared to the negative IgG control in the (A) Lung metastatic and (B) Parental cell lines. The x-axis is the fold-change value (Kindlin-1 Lung metastatic or Kindlin-1 parental/ IgG Lung metastatic or IgG parental) and the y-axis is –log10 p-value showing statistical significance.

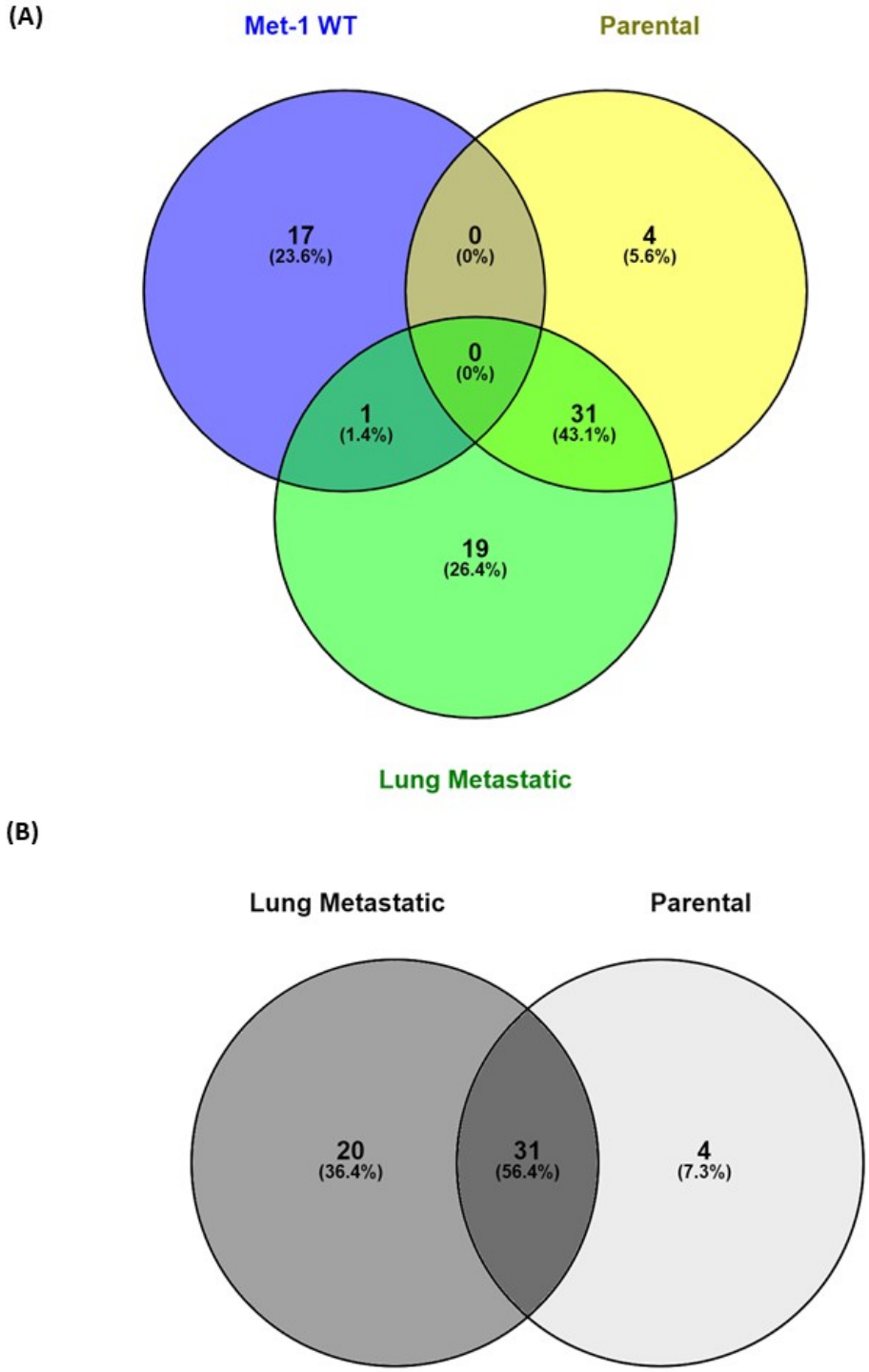


Figure 41. Venn diagrams of proteins significantly differentially bound to Kindlin-1 with an enrichment factor of 2-fold. The Met-1 Kin1-WT cell line was normalised against the Met-1 Kin1-Null cell line and the MDA-MB-231 cell lines were normalised against the IgG controls. ($p<0.05$ and enrichment factor was >2-fold) (A) Comparison of binders of Kindlin-1 in the Met-1 Kin1-WT cell line, lung metastatic variant MDA-MB-231 cell line and the parental counterpart. 18 proteins significantly differentially bound to Kindlin-1 in the Met-1 Kin1-WT cell line whilst 51 proteins significantly differentially bound to Kindlin-1 in the Lung metastatic cell line compared to 35 in the parental cell line. No common binders of Kindlin-1 were found between the three cell lines and only one common binder was found between the Met-1 Kin1-WT cell lines and the MDA-MB-231 lung metastatic cell

line. (B) Comparison of the lung metastatic variant MDA-MB-231 cell line and the parental counterpart. 31 proteins bound to Kindlin-1 in both the cell lines.

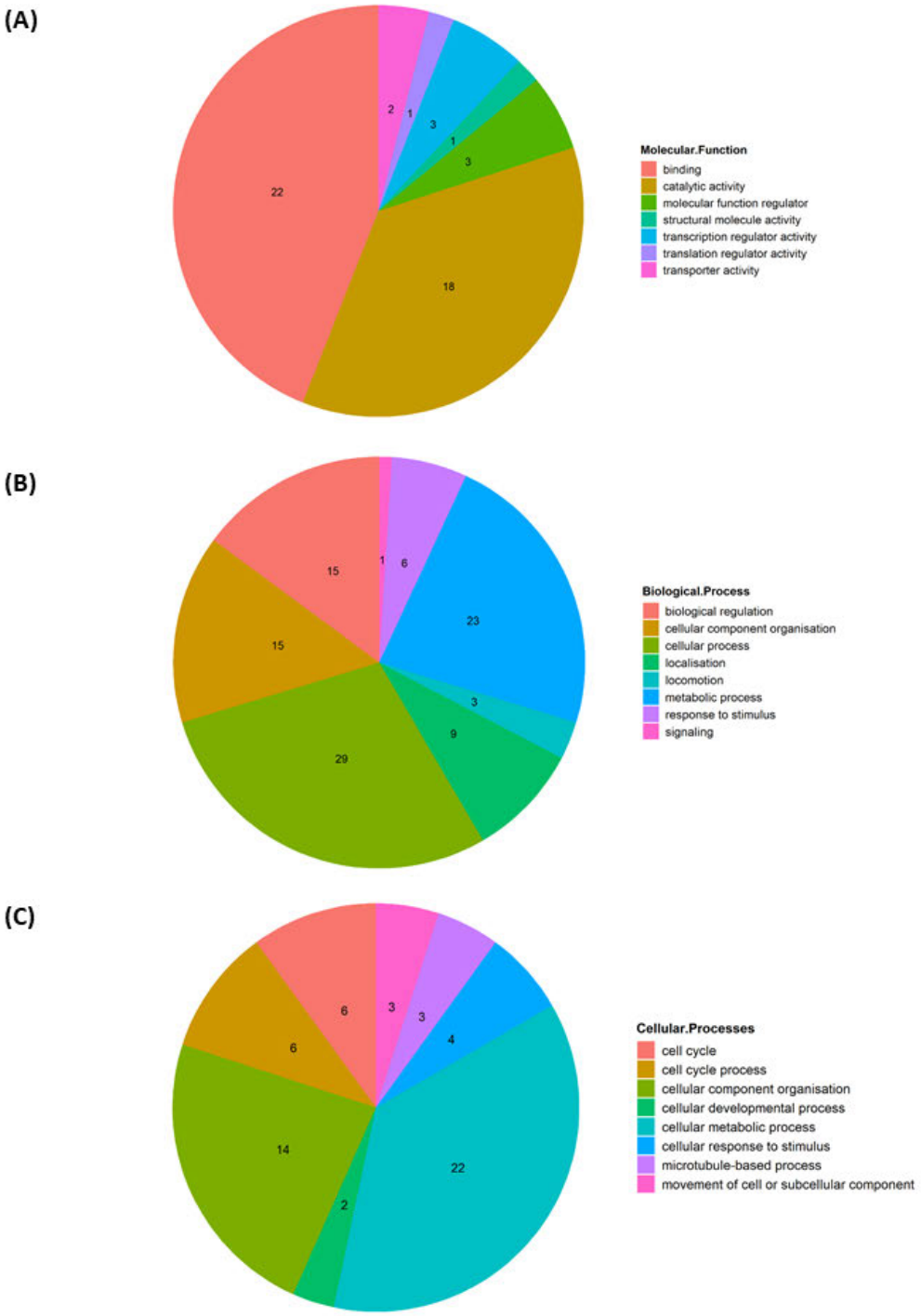


Figure 42. Gene Ontology analysis of Kindlin-1 binding proteins in Lung metastatic MDA-MB-231 cells. Pie charts show the (A) Molecular Functions, (B) Biological Processes and (C) Cellular Processes of the 51 proteins that significantly bound ($p<0.05$) to Kindlin-1 in Lung metastatic MDA-MB-231 cells with an enrichment factor of 2-fold or greater.

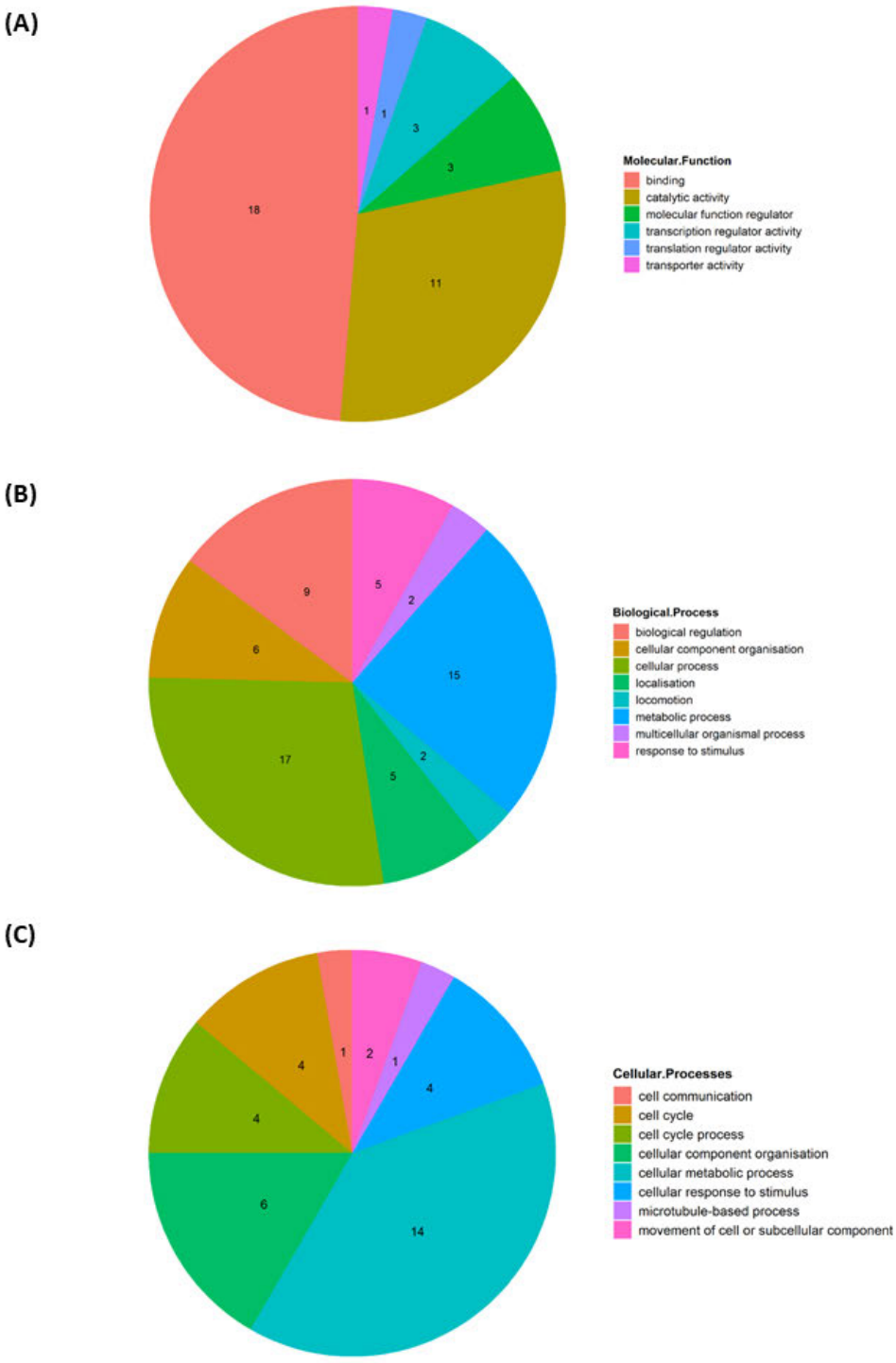


Figure 43. Gene Ontology analysis of Kindlin-1 binding proteins in parental MDA-MB-231 cells. Pie charts show the (A) Molecular Functions, (B) Biological Processes and (C) Cellular Processes of the 35 proteins that significantly bound ($p<0.05$) to Kindlin-1 in parental MDA-MB-231 cells with an enrichment factor of 2-fold or greater.

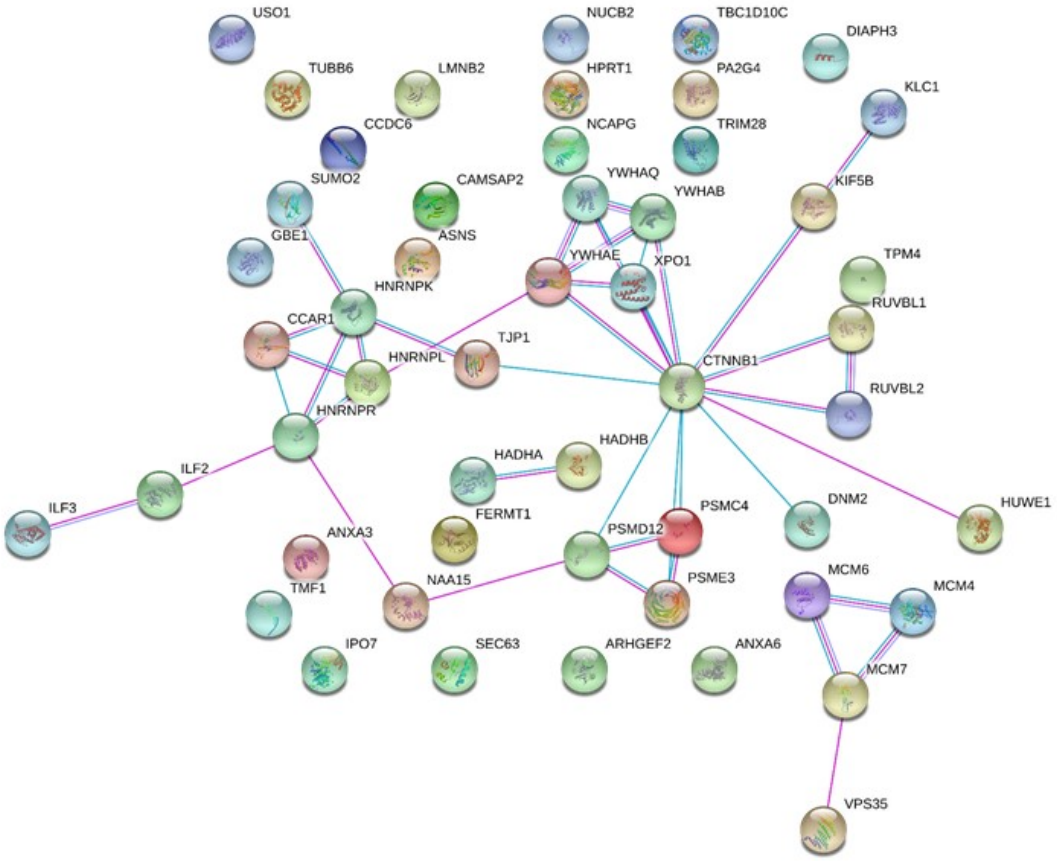


Figure 44. Lung Metastatic MDA-MB-231 Kindlin-1 interactome. A Students t-test ($p<0.05$) identified that 51 proteins significantly bound to Kindlin-1 with an enrichment factor of 2-fold or greater. Network analysis was performed using STRING.

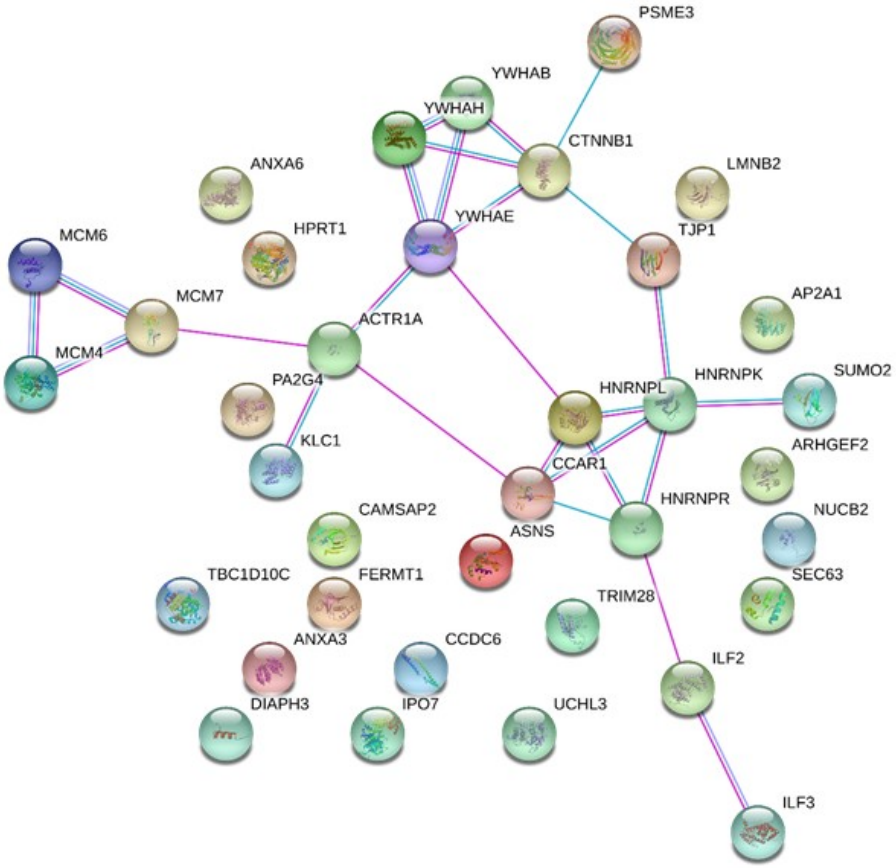


Figure 45. Parental MDA-MB-231 Kindlin-1 interactome. A Students t-test ($p<0.05$) identified that 35 proteins significantly bound to Kindlin-1 with an enrichment factor of 2-fold or greater. Network analysis was performed using STRING.

4.3 Discussion

4.3.1 In the absence of Kindlin-1, ILK is still recruited to the FAs in Met-1 and SCC cancer models

Consistent with studies using CHO cells, we found that ILK binds to Kindlin-1 in Met-1 (Fig. 30A) and SCC cells (Fig. 30B). Unlike Kindlin-2, which has been previously shown to prevent localisation of ILK to FAs upon its depletion (134), Kindlin-1 loss was not found to affect localisation of ILK to the FAs of cells in Met-1 (Fig. 31) and SCC cells (Fig. 32). Although both Kindlin-1 and Kindlin-2 have been found to target to FAs in most expressing cells, Kindlin-2 has been shown to have a better ability to accumulate at FAs compared to Kindlin-1 (160). Kindlin-2 has also been found to bind with more affinity to ILK compared with Kindlin-1 (160) and loss of either Kindlin-2 or ILK results in similar cell spreading defects indicating that recruitment of ILK to FAs is necessary for the function of Kindlin-2 (161,281,313). This suggests that the Kindlin-2-ILK interaction at focal adhesions may be more important compared with the Kindlin-1-ILK interaction and in the absence of Kindlin-1, presence of Kindlin-2 enables ILK to be successfully recruited to FAs. Further work would be to investigate if loss of both Kindlin-1 and Kindlin-2 completely prevents recruitment of ILK to focal adhesions and to compare the differences in cell spreading between loss of Kindlin-2 alone compared to Kindlin-1 and Kindlin-2 combined.

4.3.2 Kindlin-1 binds to proteins that are involved in microtubule-based processes

Our analysis has shed light on other proteins, which Kindlin-1 may also bind with to regulate microtubule-based processes. As Kindlin-1 is already known to regulate mitotic spindle assembly in breast cancer cells and promotes cellular survival (168), these results may provide a more detailed understanding of its role in regulating these processes. In mitosis, regulation of microtubules (MT) is important for bipolar spindle assembly, alignment of chromosomes and separation of sister chromatids. Loss of Kindlin-1 was shown to lead to abnormal spindle formation, a phenotype which is observed with defective MT regulation (168,284). We identified members of

the Tubulin family, Tubulin Beta 6 and Tubulin Beta 4B Class IVb (TUBB4B) as potential Kindlin-1 binding partners in our proteomic analysis (Fig. 36A,37D). As the tubulin family proteins are components of the microtubule cytoskeleton and perform essential cellular functions through the cell cycle, it is unsurprising that we have identified proteins from this family. This finding is also consistent with previous research from the Brunton group, which identified α-tubulin in GFP-Kindlin-1 pull downs in MDA-MB-231 cells (284).

Kindlin-1 is recruited to the centrosome through phosphorylation of threonine-30 by Plk1 and its concentration peaks during the G2/M phase (168). At the centrosome, Kindlin-1 subsequently mediates spindle formation through regulation of the cytoplasmic class II histone deacetylase 6, HDAC6, which is responsible for acetylating MTs (284). Acetylation of MTs is a direct readout of MT stability and depletion of Kindlin-1 was shown to reduce acetylated-tubulin levels, cause an increased incidence of toppled spindles and reduce cell proliferation, however, these defects could be reversed through inhibition of HDAC6 (284). Two other proteins identified in our analysis linked directly to microtubule-based processes were DYNC1H1 and VCP (Fig. 36A,37D). Cytoplasmic dynein is a highly conserved multi-subunit motor complex that moves towards the minus end of MTs and has housekeeping functions including orientation of the mitotic spindle, nuclear positioning, golgi maintenance, and endosomal dynamics (314). The complex consists of a heavy chain (DYNC1H1), an intermediate chain (DYNC1I1), a light intermediate chain (DYNLIC), and a light chain (DYNLC) (315). The complex has a long tail domain towards the N terminal of the protein that has docking sites for cargo, which includes mRNA, transcription factors, vesicles, growth factors and also adaptor proteins (314,316). The complex is transported from the cytoplasm into the nucleus, which may suggest a mechanism for the transport of Kindlin-1 to the nucleus (see next chapter – Fig. 46) (314). Cytoplasmic dynein been reported to promote tumour formation in several cancer types including colorectal, cervical and gastric cancer (316–318). In gastric cancer, DYNC1I1 was found to enhance proliferation and migration of cancer cells by

upregulating IL-6 expression and increasing p65 nuclear translocation (a member of the NF-kB family) (316). As our group has previously shown differential expression of IL-6 in Met-1 Kin1-Null and Met-1 Kin1-WT cells (Fig. 25A), we would expect more expression of nuclear p65 in cells with increased IL-6 expression and nuclear p65 translocation has been followed up in chapter 5 (Fig. 52). Our validation experiments to confirm binding of Kindlin-1 to Dynch1h1 were inconclusive and further optimisation was required using different beads and conditions to confirm this interaction.

We chose to follow up binding of Kindlin-1 to VCP in the Met-1 model as increased expression of VCP has been identified as a marker of poor prognosis in breast carcinoma as well as patients with cancer of the liver, stomach, prostate, colorectum, esophagus and lung (319–323). In HeLa cells, depletion of VCP prevented cell-cycle progression and was linked to mitotic abnormalities and apoptosis (324). VCP has also been shown to promote metastasis in osteosarcoma via an anti-apoptotic effect on cells (325). VCP expression lead to degradation of p-IKBα in the cytoplasm, enabling NF-kB to translocate into the nucleus and act as a protective factor against apoptosis leading to increased metastatic potential (325). In prostate cancer, IL-6 was shown to induce expression of VCP and lead to increased cell proliferation, migration, and invasion (326). As regulation of VCP is linked with IL-6 expression and our group has previously shown significantly different levels of IL-6 expression between the Met-1 Kin1-Null and Kin1-WT cells, we used the co-immunoprecipitation to validate this interaction, however, VCP appeared to be a contaminant and not a real Kindlin-1 interactor.

4.3.3 Kindlin-1 binds to proteins that are important regulators of cell cycle progression

Kindlin-1 has been reported to have a role in a number of different cellular phenotypes including cell proliferation in epithelial cells and these roles relate to the

localisation of Kindlin to FAs where it acts as an adaptor protein and helps to link the actin cytoskeleton to the ECM (118,177,327,328). Kindlin-1 loss has been reported to impair cell proliferation *in vitro* and *in vivo,* which has been linked to the phenotype of the skin in KS patients (149,177). In keratinocytes, Kindlin-1 has been shown to bind key regulators of cell cycle progression: CDK1 and CDK2 (137). In KS keratinocytes (which lack Kindlin-1), inhibition of CDK led to oxidative damage induced cell cycle arrest and DNA damage (137). In our analysis, we found Kindlin-1 bound to MCM5 and MCM6 of the MCM family in Met-1 cells (Fig. 28A). As phosphorylation by kinases (including the CDK family) is imperative for tight regulation of the MCM family in DNA replication and cell cycle progression, these Kindlin-1 binding partners may be relevant to the Kindlin-1-CDK literature (329). Moreover, the adaptor protein, ILK, which we have shown to bind Kindlin-1 in the Met-1 model, interacts with MCM7 and suppresses cell growth (330). Kindlin-1 was found to bind to MCM proteins in both mouse and human models (Fig. 36A,44,45), suggesting that this family of proteins is universally important amongst these cell lines. Knockdown of the MCM proteins has already been shown to decrease cancer cell proliferation and the MCM proteins can also be inhibited by targeting their helicase activity (331,332), highlighting this family of proteins as potential cancer therapeutic targets. As an interaction with MCM proteins was identified in all 3 data sets (Fig. 36,44,45), IHC could be performed on tumours to look at expression of these proteins in future experiments. Notably, Met-1 cells do not appear to grow differently *in vitro* and we previously reported a difference in cell proliferation *in vitro* between the Met-1 cell lines but only at the final time point of a cell counting assay (211). An *in vitro* proliferation assay could be used to assess the effect of loss of Kindlin-1 in the MDA-MB-231 lung metastatic cell line once the CRISPR-knockout has been confirmed.

4.3.4 Other relevant interactors of Kindlin-1

In our analysis of the MDA-MB-231 data sets, we identified proteins from the 14-3-3 family that are known to interact with multiple proteins that are involved in signal transduction, apoptosis, cell cycle and cell migration (Fig. 44,45) (333). The 14-3-3 family are overexpressed in different cancer types and research has shown they may have a key role in regulation of pathways that are linked to cancer initiation and progression (334). The mechanism of regulation of target proteins is primarily through phosphorylation of serine and threonine residues (335) and research has also shown that they interact with α3β1 integrins in keratinocytes to promote cell adhesion and motility (333), which could be relevant to their interaction with Kindlin-1 as a known binder of β1-integrin (Fig. 7).

Another protein of interest that was identified in both MDA-MB-231 data sets was β-catenin, a significant molecule in Wnt signalling (Fig. 44,45). It has already been reported that Kindlin-2 directly binds to active β-catenin, prevents its degradation and enables recruitment of TCF4 (170). This tripartite nuclear transcriptional complex promotes Wnt target gene expression, which enables tumour cell invasion (170). In contrast, Kindlin-1 supresses Wnt signalling, loss of Kindlin-1 was shown to induce Wnt ligand transcription and expression of Wnt target genes (156). The mechanism of suppression on the Wnt pathway was thought to be through retention of transcriptional cofactors in the cytoplasm independent of integrin (171).

Notably, we did not find common binders between all the three cell lines analysed (Fig. 33A) but we did find interactors from the same family of proteins (Fig. 36,44,45). HADHA was the only one common binder between the Met-1 Kin1-WT and the MDA-MB-231 lung metastatic cell lines (Fig. 36,41A,44). Overexpression of this enzyme has an inhibitory effect on tumour growth in clear cell renal carcinoma mechanistically by disrupting lipid metabolism (299).

4.3.4 Summary and future work

In summary, we have shown that loss of Kindlin-1 does not affect ILK recruitment to FAs and further research to investigate Kindlin-2 loss versus combined loss of Kindlin-1 and Kindlin-2 are necessary in order to observe recruitment and compensatory mechanisms within the cell. Our findings suggest that Kindlin-1 is an adaptor protein in the cell that regulates a number of key signalling hubs, which control several cellular processes including metabolism, cell proliferation, cell migration (through regulation of MTs), apoptosis and signal transduction. Other approaches could be used to compliment the Kindlin-1 binding partner data here such as BioID, which is a technique that uses biotin ligase to biotinylate proteins based on proximity to the protein of interest in living cells (336). The ligase is coupled to the protein of interest and biotinylates proximal endogenous proteins that can then be selectively isolated and identified (336). Advantages of using this technique are that it can be used for insoluble proteins and can identify weak or transient protein interactions. BioID can also be used to identify interactions with proteins that are also only expressed at a certain time (336). Both immunoprecipitation and BioID can detect proteins that are not direct binders of the protein of interest but are part of multi-protein complexes. This could be the case for some of the tubulins we have identified as Kindlin-1 interactors. It has been previously shown that Kindlin-1 does not directly bind α-tubulin, Kindlin-1 binds to Plk1 directly and to alpha-tubulin as a part of a complex (284). In order to determine whether the interaction is direct, a pull down assay using a fusion tagged bait protein (such as a Glutathione-S-transferase pull down assay) would need to be used (337). Furthermore, to identify the functional significance of proteins that bind to Kindlin-1, the binding sites of direct Kindlin-1 interactors would need to be mapped through generation of Kindlin-1 mutants and intervention studies carried out to determine if loss of the interaction of Kindlin-1 to a binding partner has an effect on tumour phenotypes.

Chapter 5 The Role of Kindlin-1 in the Nucleus of Cancer Cells

A number of FA proteins are now recognised to have functions within the nucleus; Zyxin was the first focal adhesion that was shown to shuttle from the cytoplasm into the nucleus (338) and since, Paxillin (339), ILK (340), FAK (341) and Src (342–344) among others have been shown to have roles in the nucleus. A characteristic of many FA proteins that shuttle to nucleus is the presence of LIM domains and this is also a structural feature of many nuclear proteins that control gene expression (345). By shuttling to the nucleus, FA proteins facilitate communication between two subcellular domains and it has been hypothesised that some FA proteins may have evolved to have scaffolding roles distinct from their adhesion functions. For example, nuclear FAK has a scaffolding role in the nucleus, which facilitates cell survival. Nuclear FAK inactivates p53 through binding to p53 via its FERM domain and this causes Mdm2-dependent p53 degradation (341). Several other FA proteins have also been shown to regulate hormone receptor signalling and impact gene transcription (346).

To date, Kindlin-2 is the only Kindlin family member with a recognised NLS, which is located at amino acids 55-72 (117). Kindlin-2 has been shown to localise to the nucleus in leiomyosarcomas and leiomyomas (117,191) and in mouse muscle cells and breast cancer cells, promote the transcription of genes that activate Wnt signaling (169,170).–Kindlin-2 has also been shown to repress microRNAs and promote breast cancer invasion (191). Mechanistically, nuclear Kindlin-2 binds DNA methyltransferase 3 alpha (DNMT3A) and forms a complex that occupies the promoter of miRNA-200b to repress its expression and this enables Zinc Finger E-box-binding homeobox 1 (ZEB1) to accumulate, which leads to EMT and cell invasion (191). As Kindlin-1 has previously been found in the nucleus of keratinocytes (118) and we have shown that Kindlin-1 regulates *Tnc* gene expression (185), we asked if Kindlin-1 has an important role in the nucleus to influence gene expression in breast cancer cells.

Specific aims of this chapter include:

1. Confirm the presence of Kindlin-1 in the nucleus of breast cancer and squamous cell carcinoma cell lines.
2. Identify if loss of Kindlin-1 affects localisation of other focal adhesion proteins to the nucleus of breast cancer and squamous cell carcinoma cell lines.
3. Generate a Kindlin-1 nuclear mutant that cannot localise to the nucleus for phenotypic analysis of the effect that loss of Kindlin-1 recruitment into the nucleus has *in vivo*.
4. Investigate the effect of a Kindlin-1 NLS mutant on the TNFα-p65-IL6 pathway.

5.1 Kindlin-1 and other focal adhesion proteins are found in the nucleus of Met1 and SCC cells

Cellular fractionation was performed in order to extract the nuclear proteins from the cell (Fig. 46A). In the fractionation protocol used, three fractions were collected; the cytoplasmic, perinuclear and nuclear fraction. Before blotting for proteins of interest, the fractions were checked for contaminating organelles by western using antibodies that were picked based on their compartmentalisation. GM130 was used as a marker of the perinucleus, α-tubulin was used as a cytoplasmic marker and Histone H4 was selected as a marker of the nucleus (Fig. 46A). Kindlin-1 was found in the nucleus of Met-1 Kin1-WT and Met1 Kin1-AA cells but as expected, not found in Kin1-Null cells (Fig. 46).

In addition to Kindlin-1, the focal adhesion proteins: Kindlin-2, ILK and FAK were probed for in the nucleus of Met-1 cells (Fig. 47A). Western blotting confirmed that Kindlin-2 and ILK are present in the nucleus of all three cell lines, whereas FAK was only found in the perinuclear and cytoplasmic fractions (Fig. 47A). These focal adhesion proteins were also probed for in cellular fractions of SCC cells (Fig. 48). Western blotting confirmed that Kindlin-1 was present in the nucleus of SCC Kin1-WT and Kin1-AA cells but not Kin1-Null cells, and ILK was present in the nucleus of all three cell lines (Fig. 48). Kindlin-2 and FAK were found in the perinuclear and cytoplasmic fractions but not the nuclear fractions of the SCC cells (Fig. 48). Notably, the levels of ILK found in the nucleus of the SCC Kin1-WT and Kin1-AA cell lines were higher than the levels found in the Kin1-Null cells, however, due to time constraints the experiment was not repeated in order to carry out statistical analysis (n=1) (Fig. 48).

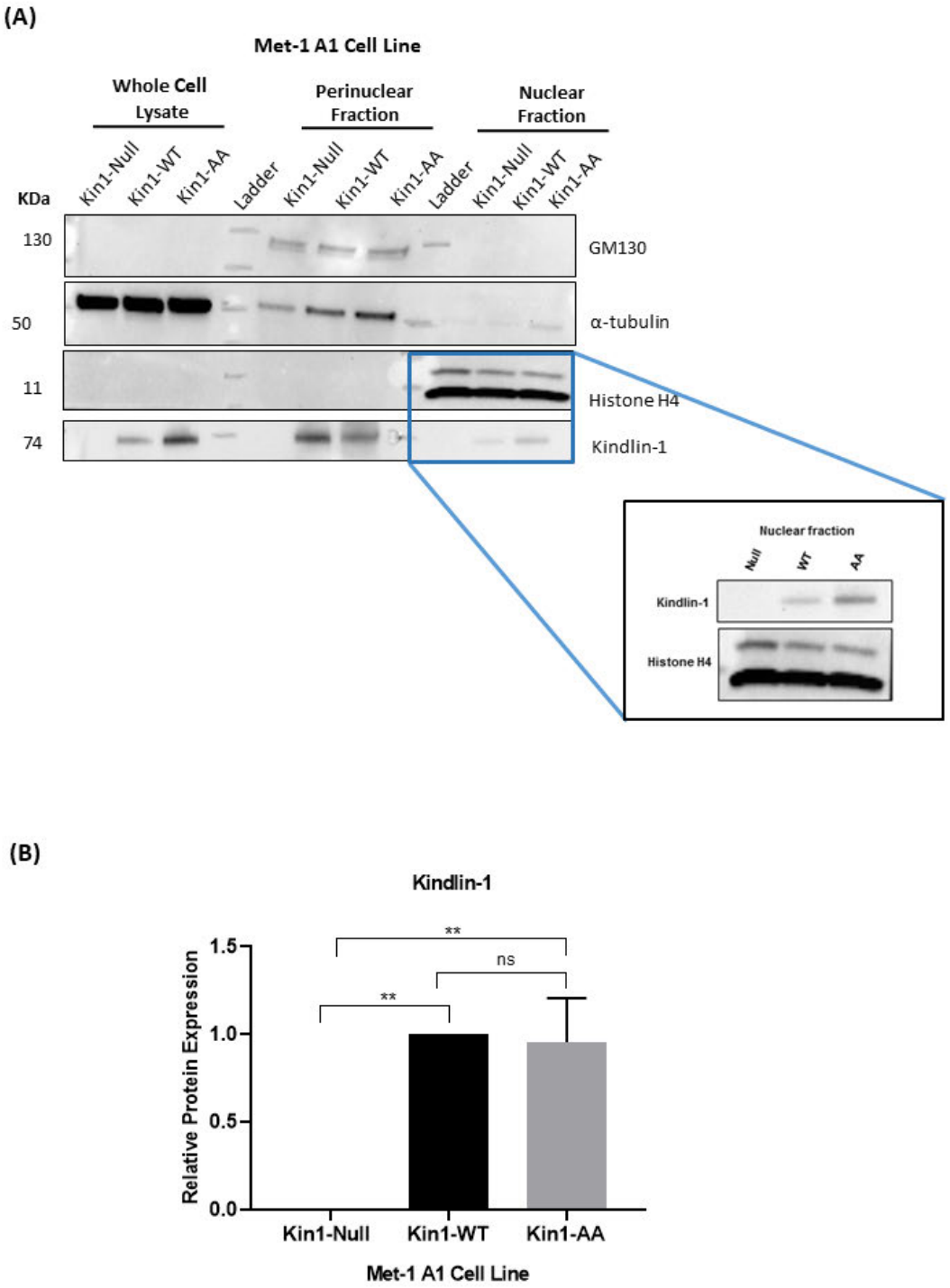


Figure 46. Kindlin expression in the nucleus in cancer cells. (A) Kindlin-1 is expressed in the nucleus and localisation to the nucleus is independent of integrin binding in Met-1 cells. Western blot analysis for Kindlin-1 in the nucleus of Met-1 Kin1-Null, Kin1-WT and Kin1-AA cells. GM130 was used as a marker of the perinucleus, α-tubulin as a cytoplasmic marker and histone H4 was used as a nuclear marker. (B) Relative Kindlin-1 protein expression in the nucleus of Met-1 cells normalised to Histone H4 (*p<0.05). Values are mean ±SEM of 3 independent experiments. P values were obtained by a one-way ANOVA.

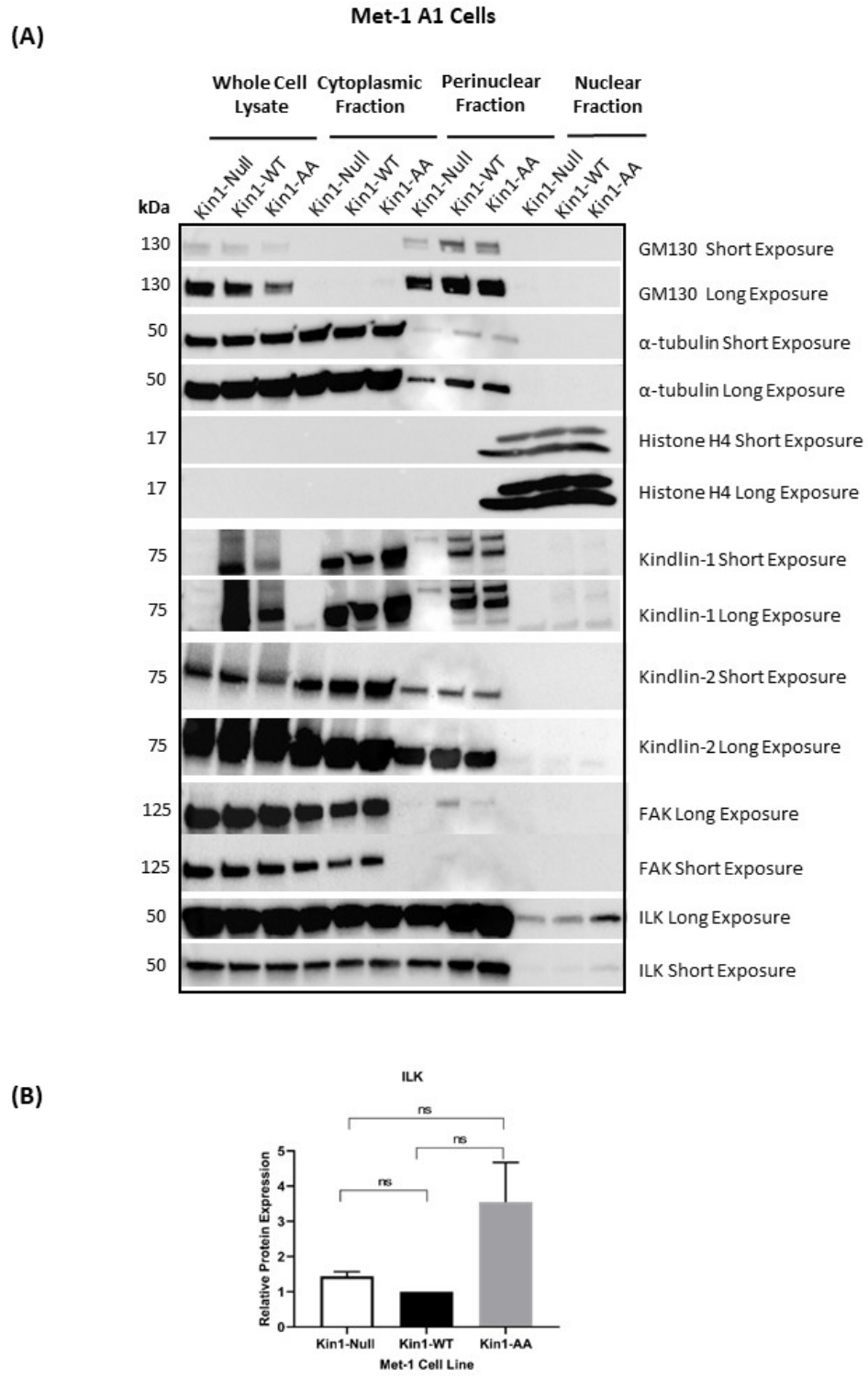


Figure 47. Focal adhesion protein expression in the nucleus of Met-1 cells. (A) Western blot analysis for Kindlin-1, Kindlin-2, FAK and ILK in the nucleus of Met-1 A1 Kin1-Null, Kin1-WT and Kin1-AA cells. Kindlin-2 and ILK are expressed in the nucleus and localisation to the nucleus is independent of presence of Kindlin-1. GM130 was used as a marker of the perinucleus, α-tubulin as a cytoplasmic marker and histone H4 was used as a nuclear marker. (B) ILK protein expression was analysed by Fiji; values are mean ± SEM of three replicates. Protein expression was normalised Kin1-WT cells. P values were obtained by a one-way ANOVA.

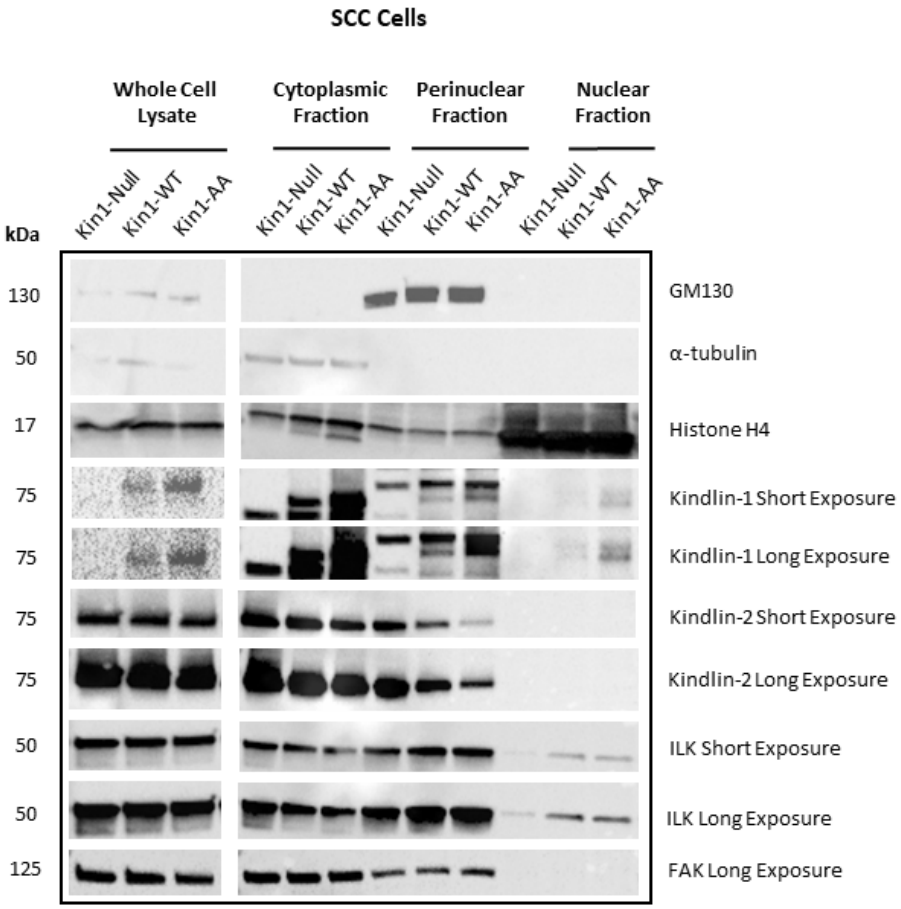


Figure 48. Focal adhesion protein expression in the nucleus of SCC cells. Western blot analysis for Kindlin-1, Kindlin-2, FAK and ILK in the nucleus of SCC Kin1-Null, Kin1-WT and Kin1-AA cells. ILK was expressed in the nucleus and localisation to the nucleus is independent of presence of Kindlin-1. GM130 was used as a marker of the perinucleus, α-tubulin as a cytoplasmic marker and histone H4 was used as a nuclear marker.

Kindlin-1 and Kindlin-2 have been shown to have both distinct and overlapping roles and examples of redundancy have been described in the literature (153–155). Overlapping functions in sustaining epithelial integrity have previously been reported with cells able to compensate for loss of Kindlin-1 through regulation of Kindlin-2 gene expression and vice versa (154). Moreover, Kindlin-1 and Kindlin-2 were found to predominantly reside in different cellular locations, however, Kindlin-1 was shown to relocate in the absence of Kindlin-2 and compensate for its functions *in vitro* (155). Loss of Kindlin-1 had no effect on ILK expression in the Met-1 model (Fig. 47B), however, Kindlin-2 was found in the nucleus of the Met-1 model (Fig. 47A). This data, combined with the redundancy that has been previously observed with Kindlin-1 and

Kindlin-2, lead us to question whether presence of Kindlin-1 is important for the import of ILK into the nucleus in the SCC model because the SCC cells lack Kindlin-2 in the nucleus (Fig. 48). We carried out preliminary experiments using Kindlin-2 siRNA in the Met-1 model to knockdown Kindlin-2 expression and establish if this impacted the amount of ILK found in the nucleus of Met-1 Kin1-Null cells compared to Kin1-WT cells, however, these experiments were inconclusive and required further optimisation.

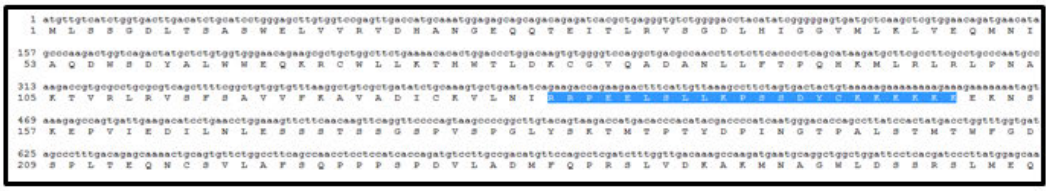
5.2 Generation of a Kindlin-1 NLS mutant that cannot localise to the nucleus

In order to test the importance of localisation of Kindlin-1 to the nucleus of cancer cells, we generated a Kindlin-1 NLS mutant (Kin1-NLS) that sequesters Kindlin-1 in the cytoplasm (Fig. 49). The Kindlin-1 NLS mutant was generated using the Met-1 CRISPR-cas9 Kindlin-1 knockout clone, clone 3B. This is a different Met-1 CRISPR-cas9 Kindlin-1 knockout clone to the clone used for the experiments in chapters 3 and 4 (clone A1). The Kin1-NLS mutant was generated using the vector, pWZL, as this was the vector used to generate the Met-1 Kin1-WT and Kin1-AA cell lines. The Met-1 Kin-1 Null Clone A1 had an empty vector pWZL re-expressed (as a control) and therefore, was resistant to the selection marker. The parental Met-1 Kin-1 Null Clone A1 was not present in frozen stocks and therefore, we infected the Met-1 3B Kin1-Null clone with the Kin1-NLS to keep the vectors containing the Kindlin-1 sequences consistent.

An NLS typically consists of one or more short sequences of basic amino acids, such as lysine and arginine (12), and using NLS mapper (347), this allowed identification of a putative NLS in the Kindlin-1 sequence (Fig. 54A). Nuclear import requires importin α, which recognises and binds to the cargo containing an NLS in the cytoplasm (348). The cargo is then linked to β-karyopherin and importin-β, to form a tripartite complex, which can interact with the nuclear pore to enable translocation to the nucleus (348). NLS peptides bind to major and minor pockets of importin-α and the import of cargo into the nucleus depends on the affinity of the NLS for

importin-α (349). Amino acids of the NLS sequence bind to the NLS-binding pockets of importin-α in an extended conformation and the main chains of the amino acids in the NLS run anti-parallel to the chain of importin-α (349). The binding pockets contain conserved tryptophans and a number of invariant asparagines four residues downstream (350,351). The lysine side chains of the NLS sequence sit in between stacked hydrophobic indole side chains of conserved tryptophans of importin-α and form salt bridges with negatively charged amino acids lining the binding pocket (350,351). The asparagine's of importin-α form key chain contacts with the NLS sequence (350,351). Therefore, in order to abrogate localisation to the nucleus, two arginine and four lysine residues in the NLS sequence, which carry a positive charge were mutated to alanine, which has no charge (Fig. 49B). The Kindlin-1 NLS mutant sequence with six amino acid mutations (NLS-4) was successfully made and confirmed by cDNA sequencing, however, retroviral infection of Kin1-NLS into Met-1 Kin1-Null cells using Phoenix-ECO cells was unsuccessful. The Met-1 Kin1-Null cells appeared unable to tolerate the amount of mutations made to the Kin1-WT sequence as the cells did not survive selection, whereas retroviral infection with Kin1-WT and Kin1-AA sequences that were performed alongside as controls were successful. The same result was found upon infection of cells with a mutant Kindlin-1 sequence containing five mutations (NLS-3) (Fig. 49B). Subsequently, mutant Kindlin-1 sequences with three (Kin1-NLS1) and four (Kin1-NLS2) mutations to the NLS sequence were infected into Met-1 Kin1-Null cells (Fig. 49B) and these mutations successfully prevented localisation of Kindlin-1 to the nucleus and perinucleus of the cells (Fig. 49C). Loss of Kindlin-1 localisation to the nucleus of the cells did not change the cell morphology (Fig. 49D).

(A)

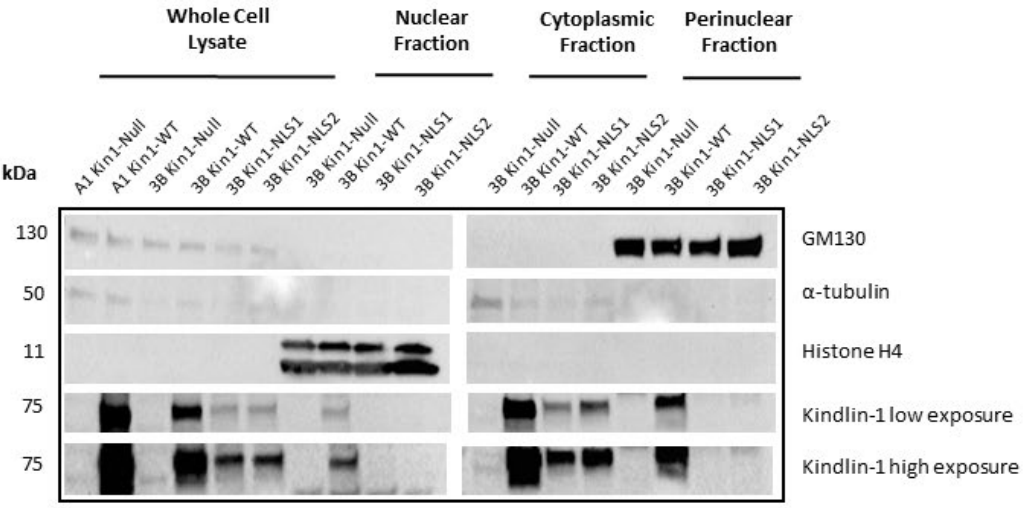


(Dr Hitesh Patel)

(B)

NLS-1	RRPEELSLLKPSSDYCKKKKKK	3 mutations
NLS-2	RRPEELSLLKPSSDYCKKKKKK	4 mutations
NLS-3	RRPEELSLLKPSSDYCKKKKKK	5 mutations
NLS-4	RRPEELSLLKPSSDYCKKKKKK	6 mutations

(C)



(D)

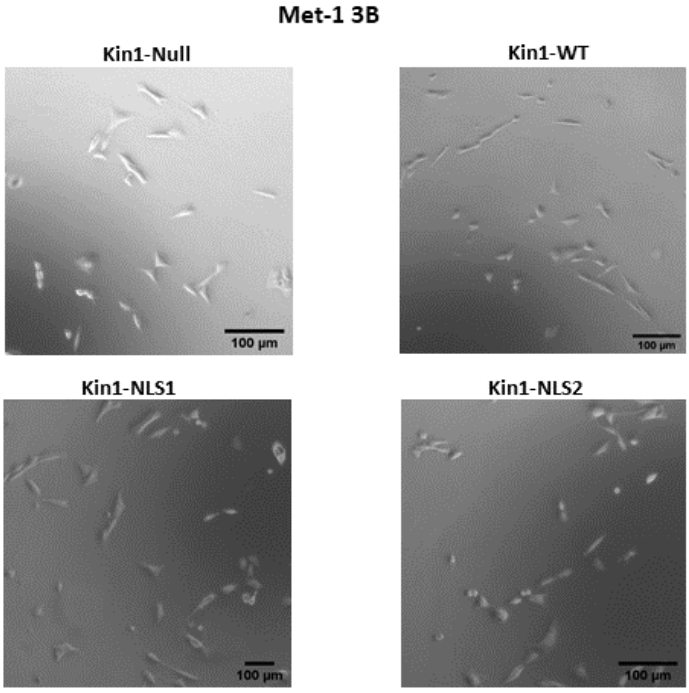


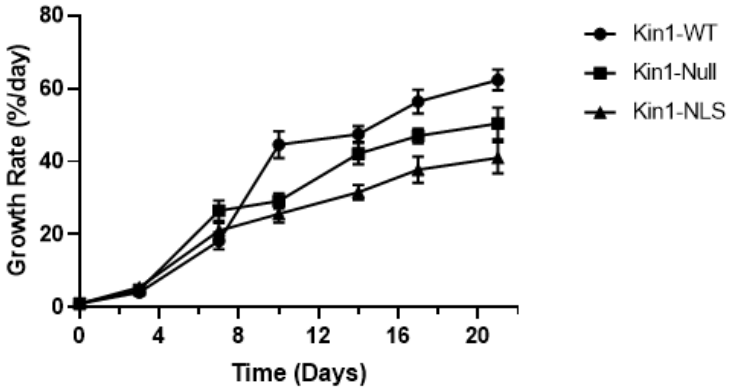
Figure 49. Generation of a Kindlin-1 nuclear localisation mutant. (A) The Kindlin-1 sequence with the predicted nuclear localisation signal (NLS) highlighted in blue (Dr Hitesh Patel) (B) Amino acids mutated to alanine in the predicted Kindlin-1 NLS sequence are shown in red. (C) Western blot analysis for Kindlin-1 in the nucleus of Met-1 clone 3B cells with genetic deletion of *Fermt1* (Kin1-Null) and re-expression of WT Kindlin-1 (Kin1-WT), Kindlin-1 nuclear localisation mutant 1 (NLS1) or Kindlin-1 nuclear localisation mutant 2 (NLS2). Kindlin-1 was not present in the nucleus or perinucleus of Met-1 3B Kin-1 Null, Kin1-NLS1 or Kin1-NLS2 cells. GM130 was used as a marker of the perinucleus, α-tubulin as a cytoplasmic marker and histone H4 was used as a nuclear marker. (D) Representative images of Met-1 clone 3B Kin1-Null, Kin1-WT, Kin1-NLS1 and Kin1-NLS2 cells. Objective magnification: 10x. Scale bar represents 100µm.

5.3 Characterisation of the effect of a Kindlin-1 NLS mutant on tumour growth

To investigate the effect that loss of Kindlin-1 localisation to the nucleus has *in vivo,* mice were inoculated subcutaneously with the Met-1 3B model (Fig. 50). The growth rate of tumours between the different cell lines did not differ until day 10 where the growth rate of Kin-1 NLS cells and Kin1-Null cells was significantly lower compared to Kin1-WT cells (Fig. 50). The growth rate of Kin1-NLS tumours was significantly lower than Kin1-WT tumours at each time point from day 10 (Fig. 50). Although the growth rate of Kin1-Null tumours was lower than Kin1-WT tumours after day 10, the

difference in growth rate at each time point did not reach significance (Fig. 50). Kin1-NLS tumours had a consistently lower growth rate compared to Kin1-Null tumours from day 10 onwards but the growth rate was not significantly lower at each time point (Fig. 50).

(A)



(B)

Time (Days)	Comparison	P value	Summary
3	Kin1-WT vs. Kin1-Null	0.7402	ns
3	Kin1-WT vs. Kin1-NLS	0.2368	ns
3	Kin1-Null vs. Kin1-NLS	0.4814	ns
7	Kin1-WT vs. Kin1-Null	0.1123	ns
7	Kin1-WT vs. Kin1-NLS	0.6825	ns
7	Kin1-Null vs. Kin1-NLS	0.2603	ns
10	Kin1-WT vs. Kin1-Null	0.0221	*
10	Kin1-WT vs. Kin1-NLS	0.0028	**
10	Kin1-Null vs. Kin1-NLS	0.4222	ns
14	Kin1-WT vs. Kin1-Null	0.2319	ns
14	Kin1-WT vs. Kin1-NLS	0.0057	**
14	Kin1-Null vs. Kin1-NLS	0.1062	ns
17	Kin1-WT vs. Kin1-Null	0.1229	ns
17	Kin1-WT vs. Kin1-NLS	0.0055	**
17	Kin1-Null vs. Kin1-NLS	0.0970	ns
21	Kin1-WT vs. Kin1-Null	0.1555	ns
21	Kin1-WT vs. Kin1-NLS	0.0086	**
21	Kin1-Null vs. Kin1-NLS	0.2668	ns

Figure 50. Tumour growth with loss of Kindlin-1 localisation to the nucleus. (A) Mean (±SEM) tumour growth of mice inoculated with Met-1 3B Kin1-Null, Kin1-WT or Kin1-AA cells (n=16 tumours per group). (B) Table displaying results of a two-way ANOVA of growth rates of mice inoculated with Met-1 Kin1-Null, Kin1-WT or Kin1-AA cells. Row number corresponds to each time point. (n=10 tumours per group, *p<0.05, **p<0.01, one-way ANOVA).

5.4 General impact of a Kindlin-1 NLS mutant on the TNFα-p65-IL6 pathway

It has been previously shown that DYNC1I1, a component of the dynein multi-protein complex, increases proliferation and migration of tumour cells in gastric cancer by increasing p65 nuclear translocation and the expression of IL-6 (316). As data suggested that Kindlin-1 binds a subunit of cytoplasmic Dynein, Dync1h1, in the Met-1 Kin1-WT cell line (Fig. 36A,37D) and previous data showed an increase in IL-6 with the loss of Kindlin-1 (Fig. 25A), we firstly examined *Il-6* expression in the Met-1 3B clone (Fig. 51). There was a small increase in *Il-6* expression compared to Kin1-WT cells (Fig. 51) but this did not reach statistical significance, which was consistent with what we observed in previous experiments using the A1 clone (Fig. 25E). Gene expression analysis showed that *Il-6* expression did not significantly differ between the Kin1-Null and Kin1-NLS cells (Fig. 51).

As translocation of p65 to the nucleus is known to regulate *IL6* expression, we also investigated nuclear p65 protein levels in these cell lines under basal conditions and after incubation with the inflammatory cytokine, Tumour Necrosis Factor alpha (TNFα), to stimulate p65 nuclear translocation. TNFα is a pro-inflammatory cytokine that stimulates the canonical NF-κB pathway, which leads to activation of p65 (otherwise known as RelA) containing dimers (352,353). The dimer translocates to the nucleus once activated where it regulates pro-inflammatory and cell survival genes, which include IL-6 (352,353). There was little nuclear p65 under basal conditions but when stimulated with TNFα, Met-1 Kin1-WT cells had a higher level of nuclear p65 compared to Kin1-Null and Kin1-NLS2 cells (Fig. 52). There was a similar increase in p65 in the perinuclear fraction in all Met-1 cell lines suggesting that Kindlin-1 may be involved in translocation of p65 to the nucleus (Fig. 52). This

experiment was only carried out once due to time constraints and therefore, statistical analysis could not be performed.

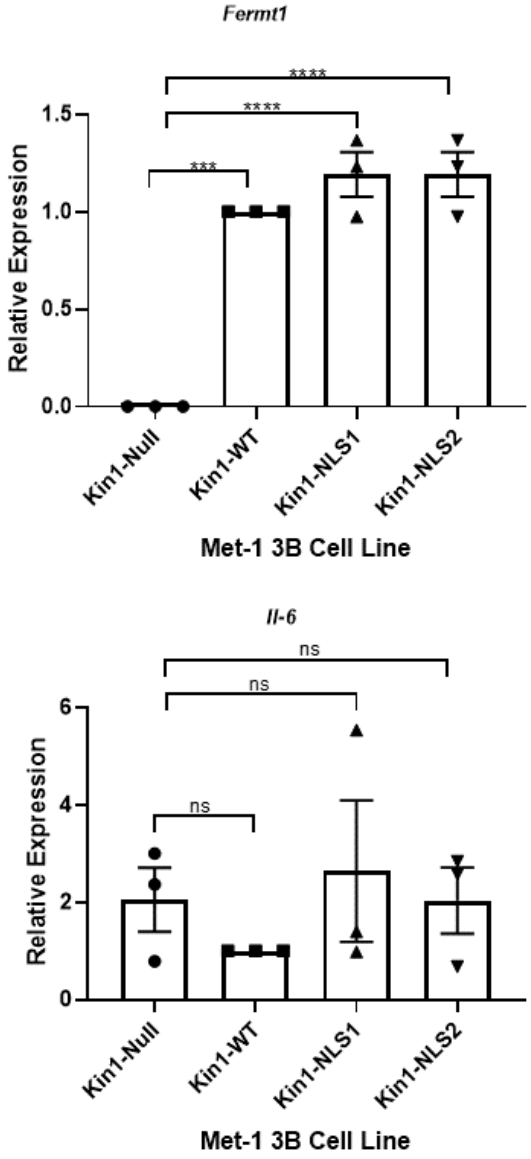


Figure 51. *Fermt1* and *Il-6* gene expression analysis in Met-1 Clone 3B cells. *Fermt1* and *Il-6* expression in Met-1 clone 3B cells with genetic deletion of *Fermt1* (Kin1-Null) and re-expression of WT Kindlin-1 (Kin1-WT), Kindlin-1 nuclear localisation mutant 1 (NLS1) or Kindlin-1 nuclear localisation mutant 2 (NLS2). Expression was analysed by qRT-PCR; values are mean ± SEM of triplicate experiments. The expression in Met-1 Kin1-Null cells was normalised to Met-1 Kin1-WT cells. (*****p*<0.0001, ****p*<0.001*).* P values were obtained by a one-way ANOVA.

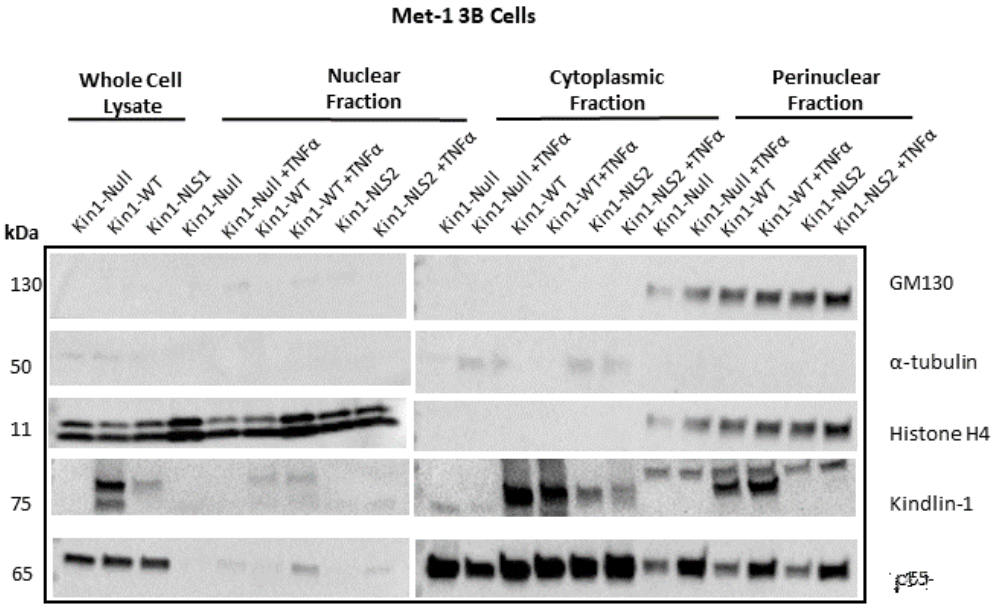


Figure 52. Loss of Kindlin-1 reduces translocation of p65 to the nucleus after stimulation with TNFα and presence of Kindlin-1 that can not localise to the nucleus does not restore the phenotype to that of Kin1-WT cells. Western blot analysis for Kindlin-1 and p65 in the nucleus of Met-1 clone 3B Kin1-Null, Kin1-WT and Kin1-NLS2 cells. Cells were stimulated with 100ng/mL TNFα for 5 hours before nuclear fractionation was performed. GM130 was used as a marker of the perinucleus, α-tubulin as a cytoplasmic marker and histone H4 was used as a nuclear marker.

5.5 Discussion

5.5.1 Kindlin-1 is found in the nucleus of two different cancer models

To our knowledge, this is the first study to report Kindlin-1 in the nucleus of cancer cell lines (Fig. 46-48). Kindlin-1 has been identified in the nucleus of normal human keratinocytes using IF (118), however, a nuclear role for Kindlin-1 in these cells has not yet been established. This study confirms that Kindlin-1 is able to localise to the nucleus in breast cancer and squamous cell carcinoma cells (Fig. 51-53). We have also shown that mutation to certain amino acids in our predicted NLS sequence, sequesters Kindlin-1 in the cytoplasm and this confirms that like Kindlin-2, Kindlin-1 has a functional NLS sequence (Fig. 49A-C). Interestingly, it was not possible to generate a Kindlin-1 nuclear localisation mutant containing five or six mutations to our predicted NLS. Despite several attempts at infecting Met-1 Kin1-Null cells with the five or six amino acid mutations to the NLS sequence, the cells were unable to

survive. As we were able to generate NLS mutants with three and four mutations to the NLS sequence (Fig. 49C), it is possible that mutating five or six amino acids of the sequence caused Kindlin-1 to misfold. Large expression of a misfolded protein could cause havoc in the cell through unspecific interactions and this may trigger apoptosis signalling. Another hypothesis is that the five and six amino acid mutant-Kindlin-1 proteins may not be able interact with the same proteins as Kindlin-1 WT or could have more or less affinity for certain proteins. If the mutations to the NLS increased the affinity of Kindlin-1 for some proteins, this could make the interactions between Kindlin and an interactor much stronger and have a kidnapping mechanism, preventing these proteins from undergoing normal cellular interactions and/or roles. Notably, the levels of the Kin1-NLS that were expressed into Met-1 Kin1-Null cells were much lower than the Kin1-WT re-expressed protein levels, which suggests that the cells are not happy with high levels of the NLS mutant (Fig. 49C).

Interestingly, the Kindlin-1 NLS mutants blocked localisation of Kindlin-1 to the perinucleus as well as the nucleus (Fig. 49C). NLS mutants strictly block importin-mediated nuclear import as described above but whether these mutants affect localisation to other parts of the cell is not well reported. Many cell fractionation protocols extract the nucleus and cytoplasmic fractions only and do not isolate a perinuclear fraction. Therefore, the NLS mutant protein would need to be tagged with a fluorescent protein to observe by microscopy where in the cell the mutant protein accumulates. On one hand, it is possible that mutation of the NLS may cause a queue of proteins outside the nucleus i.e. accumulation at the perinuclear space - on the other hand, the cell may not even try to translocate the protein if there is no functional NLS present, which is possibly why we do not see Kindlin-1 in the perinuclear fraction with the NLS mutation (Fig. 49C). There may also be differences in accumulation of the protein in the perinuclear space due to structural changes that aren't related to nuclear trafficking. Furthermore, the "perinuclear fraction" isn't strictly the best name for the perinuclear fraction isolated in the protocol we have used here as the marker chosen for the perinuclear fraction (GM130), does not exclude other vesicular structures such as the endoplasmic reticulum and

endosomes, for example. Data have shown (unpublished, Adam Byron) that some endosome subpopulations and some plasma membrane markers are present in this fraction and it is therefore more accurate to call this fraction a membrane region. As the perinuclear fraction represents vesicular structures, loss of Kindlin-1 in the perinuclear fraction could be related to transport back into the cytoplasm and because Kindlin-1 does not enter the nucleus in the first place, the protein is not transported back into the cytoplasm and is therefore, not present in vesicular structures for trafficking.

In future experiments, a wider panel of antibodies should be used to check for contaminating organelles by western after carrying out cellular fractionation experiments. In addition, mRNA could be extracted from the nucleus and RT-qPCR performed to further confirm that there is no Kindlin-1 present in the nucleus in the Kin1-NLS cell line. The Kin1-WT and Kin1-NLS proteins could also be tagged with a fluorescent protein to observe by microscopy where Kindlin-1 is accumulating in the organelles of the different cell lines.

5.5.2 Kindlin-1 and focal adhesion proteins in the nucleus

We have shown that in Met-1 cells, the focal adhesion proteins: Kindlin-2 and ILK are detected at similar levels in the nucleus in the presence and absence of Kindlin-1 (Fig. 47). Preliminary data in SCC cells showed that with loss of Kindlin-1, there was a lower level of ILK detected in the nucleus and we did not detect Kindlin-2 in the nucleus with the presence or absence of Kindlin-1 in this model (Fig. 48). ILK has a recognised NLS and is known to bind both Kindlin-1 and Kindlin-2 (117,354). Although the role of ILK in the nucleus is not well-known, ILK has been shown to shuttle to the nucleus after phosphorylation by PAK1 and regulate gene expression of the connector enhancer of kinase suppressor of Ras3 by interacting with gene regulatory chromatin (355). ILK has also been found to increase DNA synthesis in keratinocytes and was identified as a cargo protein, which binds to a carrier called Chromosomal

Maintenance 1, also known as Exportin 1 for translocation out of the nucleus and this is modulated by dephosphorylation by ILK Associated Serine/Threonine Phosphatase (ILKAP) (356). The binding of Kindlin-2 to ILK is necessary for Kindlin-2 localisation to FAs (354) and so, it is feasible that ILK and Kindlin-2 may get trafficked to the nucleus together from cell focal adhesions. As Kindlin-1 is also known to bind ILK, it is possible that the two proteins also get trafficked to the nucleus together. There is redundancy between the two proteins, which has been discussed in previous chapters and therefore, it is possible that the Kindlin-1-ILK interaction becomes more important in the absence of Kindlin-2 recruitment to the nucleus especially as ILK is known to have a stronger binding affinity for Kindlin-2 compared to Kindlin-1 (117). In SCC cells, we observed a lower level of ILK in the nucleus with loss of Kindlin-1 and we did not detect Kindlin-2 in the nucleus (Fig. 48). This suggests that when both Kindlin-1 and Kindlin-2 are absent or not recruited to the nucleus, this may result in less ILK trafficked to the nucleus. However, preliminary experiments using Kindlin-2 siRNA in Met-1 cells were inconclusive and the experiment needs further optimisation. There is already evidence for FA proteins shuttling to or from the nucleus as carrier complexes – Paxillin, which does not have a canonical NLS has been proposed to transport into the nucleus via a carrier complex and c-Abl, PABP-1 and FAK have been proposed as candidates (357–360). Furthermore, Nakrieko *et al.* (356) suggested that when ILKAP binds ILK in the nucleus, it may modify ILK or another ILK-interacting protein and enhance nuclear export. Therefore, the importance of a Kindlin-ILK interaction for nuclear transport is not impossible. As ILK is also a focal adhesion protein with important roles in tumourigenesis and there is evidence for both Kindlin-2 (169,170) and ILK (361,362) acting as transcriptional regulators in the nucleus, their interaction and the mechanism of trafficking to the nucleus is something that could be important to explore further.

Notably, FAK has previously been detected in the nucleus of other SCC models but we did not detect FAK in the nucleus of the SCC cells used in this study (Fig. 48), or in the nucleus of the Met-1 cells (Fig. 47A). In SCC cells, nuclear FAK has been shown to

have an immuno-modulatory role through the regulation of the transcription of inflammatory cytokines and chemokines including Ccl5 and TGFβ2 in a kinase-dependent manner (363). Mechanistically, nuclear FAK associates with chromatin and interacts with a network of transcription factors and their regulators, which leads to modulation of Ccl5 expression (363). Ccl5 promotes recruitment of Tregs and supresses the CD8+T cell population, which supresses the anti-tumour response of the immune system enabling tumours cells to grow (363). Furthermore, the kinase activity of nuclear FAK has also been shown to control chromatin accessibility and control gene expression of the cytokine Il33, which like Ccl5, is also involved in anti-tumour immunity (364). In addition, FAK has been shown to have adaptor functions in the nucleus, as previously mentioned, FAK associates with p53 and enhances its degradation to promote cell proliferation and survival (341). Nuclear FAK also regulates GATA4 in a kinase-independent manner – FAK binds to GATA4 via its FERM domain and regulates GATA4 through polyubiquitination (365). FAK has an NLS located in the F2 lobe of its FERM domain (341) and accumulates in the nucleus in many different cell types under conditions of cell stress (366–368), which is perhaps why we did not detect FAK in the nucleus of the SCC or Met-1 cells at basal conditions (Fig. 47,48). For future studies, the presence of focal adhesion proteins could be investigated in the cellular fractions of Kindlin-1-deficient cells under different conditions of cellular stress to observe whether their localisation to the nucleus is impacted.

5.5.3 Loss of Kindlin-1 to the nucleus significantly reduces the growth rate of primary tumours

To our knowledge, this is the first study to report a nuclear role for Kindlin-1 in tumour growth (Fig. 50). We showed that primary tumour growth is significantly reduced when Kindlin-1 is confined to the cytoplasm of cancer cells (Fig. 50). Interestingly, although the growth rate of cells with complete loss of Kindlin-1 was consistently lower than those expressing wild-type Kindlin-1, the growth rate was only significantly reduced at day 10 (Fig. 50). Interestingly, Kindlin-1-deficient

tumours from the Met-1 clone A1 model had completely regressed by day 16, whereas the growth rate of Kin1-Null tumours from the 3B clone was still exponentially increasing at day 16 (compare Fig. 24A with Fig. 50A). These models were generated from single cell clones and the data suggests that there are clonal differences. The *in vivo* study using the Met-1 3B clone needs to be repeated to establish if the Met-1 3B Kin1-Null and Kin1-NLS cells regress at a later time point or continue to grow at a reduced rate to Kin1-WT tumours.

In the SCC model of tumour growth, loss of Kindlin-1 promotes tumorigenesis (210), which suggests that Kindlin-1 has differential roles in the cellular compartments of different cell types. As previously discussed, nuclear roles of other FA proteins have been established in tumorigenesis and Kindlin-2 already has a known role in breast cancer cells. We have not established the mechanism in which Kindlin-1 regulates tumour growth in the nucleus of breast cancer cells. Notably, Rognoni *et al.* (171) reported Kindlin-1 in the nucleus of keratinocytes by IF but excluded a nuclear role for Kindlin-1 in the nucleus in regulation of Wnt signaling in these cells by expressing Kindlin-1-GFP fused to two SV40 NLS in Kindlin-1 deficient keratinocytes. It would be interesting to investigate the nuclear binding partners of Kindlin-1 to establish if Kindlin-1 forms transcriptional complexes in the nucleus or binds to DNA methyltransferase, similarly to Kindlin-2 (169,170,191), to either prevent or promote transcription of genes.

5.5.4 Regulation of p65 in the nucleus by Kindlin-1

As data suggested that Kindlin-1 binds a subunit of cytoplasmic Dynein, Dync1h1, and cytoplasmic Dynein has been previously linked to regulation of nuclear p65, we investigated what the significance of this might be by investigating if loss of Kindlin-1 impacted the amount of p65 in the nucleus. Preliminary data suggest that when stimulated with TNFα, Kindlin-1 regulates the amount of p65 in the nucleus (Fig. 52). As levels of nuclear p65 detected with loss of Kindlin-1 were similar to the levels of

nuclear p65 found in the NLS mutant and the amount of p65 found in the perinucleus was consistent between the cell lines (Fig. 52), it suggests Kindlin-1 may transcriptionally influence p65 levels. Many FAs are emerging as important regulators of gene expression by acting as adaptor proteins that bind transcription factors and as complexes, bind promoter regions of genes – as described for FAK (363) and Kindlin-2 (169,170,191). Therefore, a potential role for Kindlin-1 in the regulation of p65 in the nucleus warrants further investigation.

TNFα activates canonical NF-κB signalling, a pathway which regulates and increases the expression of IL-6 (352,353,369). Therefore, with increased nuclear p65 in Met-1 3B Kin1-WT cells, we would expect increased levels of IL-6 expression. IL-6 mRNA expression was not significantly different between the cell lines of the Met-1 3B clone (Fig. 51), and, similarly, mRNA levels did not significantly differ in the Met-1 A1 clone (Fig. 25E). As no significant difference was observed between IL-6 mRNA levels in the Met-1 cell lines, this suggests that Kindlin-1 is regulating IL-6 post-transcriptionally and therefore, we would not expect differences in the NF-κB pathway between the Met-1 Kin1-Null and Kin1-WT cells as this pathway regulates transcription of IL-6. In the Met-1 A1 clone, the protein levels of IL-6 were significantly increased with loss of Kindlin-1 (Fig. 25A) and data (not shown) from the Brunton lab has also demonstrated increased secretion of IL-6 with Kindlin-1 loss using the forward phase array. Collectively, this suggests that Kindlin-1 may affect protein stability or degradation of IL-6. It is interesting that we have identified the NF-κB pathway as being regulated by Kindlin-1, as this is novel, however, the expression of other NF-κB gene targets need to be evaluated to obtain more evidence for a role for Kindlin-1 in the regulation of the NF-κB pathway. The NF-κB pathway regulates multiple cytokines and immunoregulatory proteins that could be investigated in addition to IL-6.

5.5.4 Summary and future work

In summary, our data reveal a novel cellular location for Kindlin-1 in cancer cells. We show that Kindlin-1 is in the nucleus of breast cancer and squamous cell carcinoma cells and that Kindlin-1 has an NLS that can be altered to prevent protein localisation to the nucleus. We have performed preliminary analysis that suggests that nuclear Kindlin-1 is important for primary tumour growth and that Kindlin-1 may be important for p65 regulation in the nucleus.

For future studies, it would be interesting to perform immunoprecipitation with a Kindlin-1-specific antibody followed by mass spectrometry analysis to compare the interactome of the Met-1 Kin1-NLS with the Kin1-WT cell line. It would also be valuable to extract the nuclear fraction from these cells and perform a Kindlin-1 nuclear immunoprecipitation in order to focus on Kindlin-1 binding partners within the nucleus to drill down into its role and mechanism here, which is unknown. As phosphorylation and dephosphorylation has been shown to be important for the import or export of ILK to and from the nucleus, investigating modification of Kindlin-1, would be useful in order to establish the mechanism of Kindlin-1 translocation to the nucleus.

Immunophenotyping of the tumours from *in vivo* experiments would provide information on the status of the immune system and establish if T cells ratios are impacted by loss of localisation of Kindlin-1 to the nucleus. This would be important to establish if Kindlin-1 is transcriptionally controlling factors, which regulate the T cells. In addition, an IL-6 ELISA assay using the Met-1 3B clone would confirm if Kindlin-1 has the same effect on IL-6 protein levels in the Met-1 3B clone and whether a Kindlin-1 nuclear mutant impacts IL-6 levels. Further analysis on gene targets of the NF-κB pathway and investigation of the effect of Kindlin-1 on p65 in the nucleus of the different cell lines may also help elucidate whether Kindlin-1 has a wider role in in regulating this pathway.

Chapter 6 Concluding Remarks and Future Perspectives

The mechanisms by which Kindlin-1 mediates metastasis to the lungs in breast cancer remain relatively unknown. The Brunton group previously demonstrated that Kindlin-1 promotes lung metastasis by supporting cell adhesion in the lung endothelium through its interaction with β1-integrin and that β1 integrin is required for micrometastatic outgrowth (185). We hypothesised that additional effects may occur downstream of the adhesion defects that we observed with loss of Kindlin-1 and that Kindlin-1 may impact outgrowth or colonisation of tumour cells. This project aimed to investigate the role of Kindlin-1 in breast cancer and specifically:

- Determine if Kindlin-1 regulates outgrowth of micrometastases.
- Establish if Kindlin-1 affects breast cancer stem cell activity.
- Identify binding partners of Kindlin-1 in different breast cancer cell models.
- Explore a nuclear role for Kindlin-1 in breast cancer.

The following findings of this study contribute to existing knowledge:

- Kindlin-1 has an integrin-dependent role in tumour initiation. (Chapter 3)
- The immune system impacts on the ability of Kindlin-1 to regulate tumour growth. (Chapter 3)
- Loss of localisation of Kindlin-1 to the nucleus reduces primary tumour growth. (Chapter 5)

The functions of Kindlin-1 that we have identified and how these roles may interrelate are summarised in Figure 53.

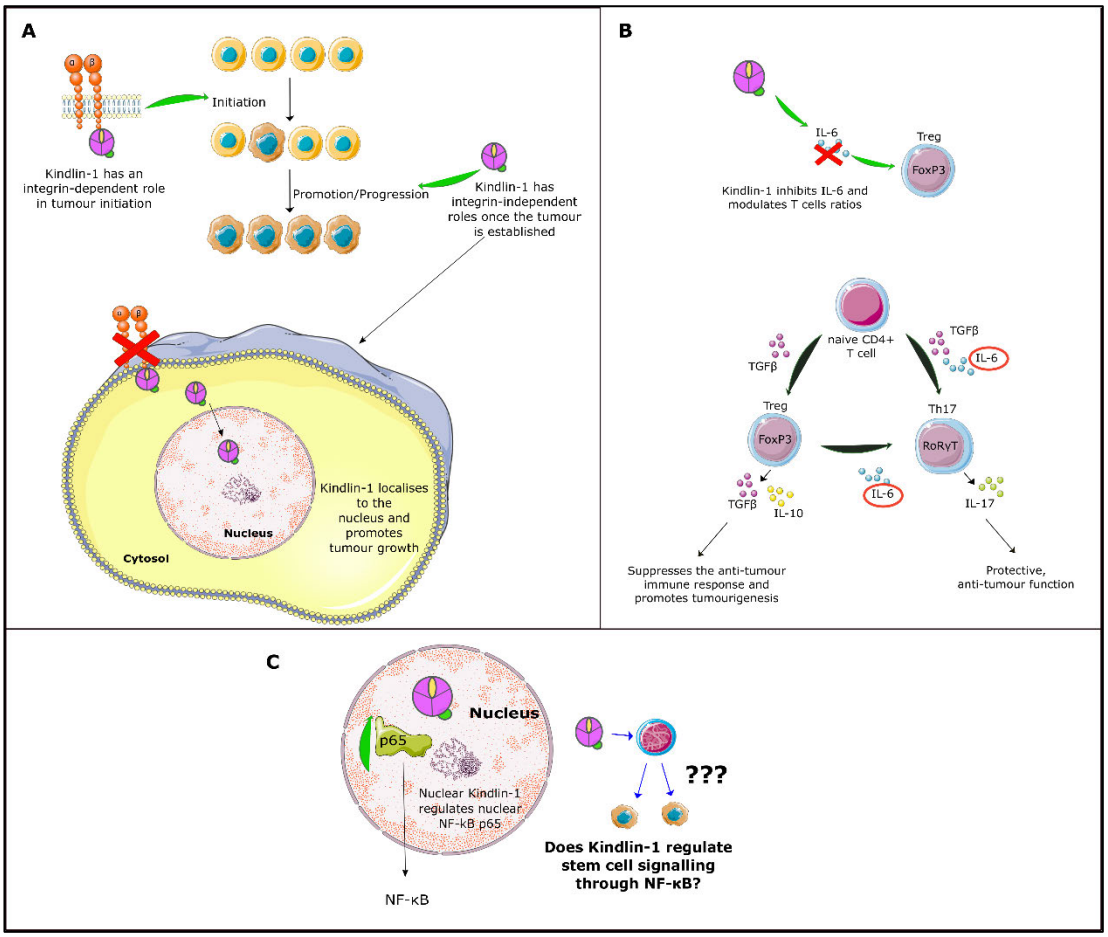


Figure 53. Summary of the mechanisms that Kindlin-1 may regulate primary tumour growth. (A) Integrin activation by Kindlin-1 is required for tumour initiation but Kindlin-1 has integrin-independent roles including within the nucleus for the promotion and progression of breast cancer. (B) Kindlin-1 modulates T cell ratios through regulation of IL-6. Kindlin-1 inhibits production of IL-6 pushing the differentiation of naïve CD4+ T cells away from T helper 17 (Th17) and towards T regulatory (Tregs) cells, which suppresses the anti-tumour immune response and leads to tumour growth. (C) Nuclear Kindlin-1 increases levels of p65 in the nucleus, a protein that dimerises with NF-κB to form a complex that functions as a transcriptional activator of NF-κB target genes. As the NF-κB pathway has a role in stem cell signalling, this raises the question: does nuclear Kindlin-1 promote tumour growth by regulating stem cell signalling via the NF-κB pathway?

6.1 A role in tumour initiation

Consistent with previous reports (186), Kindlin-1 was found to affect primary tumour growth and this was demonstrated in a mouse and human model. The Brunton group previously reported that Kindlin-1 has a role in early pulmonary metastases (185) and interestingly, we found that Kindlin-1's interaction with integrin affected early primary tumour growth following implantation of Met-1 cells. An integrin binding

deficient Kindlin-1 mutant demonstrated that early primary tumour growth was integrin dependent, however, integrin binding was not required after this stage as tumours later reached the same growth rate as the Kindlin-1 WT tumours. Activation of integrin by Kindlin-1 was also important for tumour initiation in the PyMT spontaneous tumour mouse model (178). The importance of Kindlin-1 in the initial establishment of the tumour was also suggested in our human model of Kindlin-1 loss. We found a reduction in tumour growth when MDA-MB-231 cells containing shRNA that targeted Kindlin-1 were inoculated in mice. However, we did not see a reduction in tumour growth after tumour fragments containing the shRNA Kin-1 cells were transplanted into mice. We propose that knockdown of Kindlin-1 did not impact tumour growth in the tumour fragment model as tumour fragments are established tumours that have bypassed the tumour initiation phase. Whereas in the cell inoculation model, tumour initiation was hindered with knockdown of Kindlin-1 and this led to significantly reduced tumour growth. As we have shown that Kindlin-1 is important in tumour initiation in the primary growth model, it would be interesting to establish if Kindlin-1 is equally important to establish micrometastases. The FA protein, FAK, is downstream of β1-integrin and has been associated with the regulation of initiation and progression in cancer cells. Deletion of β-integrin in a MMTV transgenic mouse model has been shown to reduce tumour burden mechanistically by reducing FAK phosphorylation and decreasing cell proliferation (239). Another *in vivo* study using mouse mammary cancer cell lines demonstrated that β1-integrin/FAK and the MAPK pathway was important for metastatic outgrowth (370). As Kindlin-1 has an important role in β1-integrin activation, the effect of Kindlin-1 on the outgrowth of micrometastases should be revisited.

As the integrins have been implicated in stem cell regulation (235–238) and tenascin-C expression was shown to be dependent on Kindlin-1-integrin interaction (185), we investigated a role for Kindlin-1 in stem cell signalling using the mammosphere assay. This assay quantifies breast stem cell activity, however, the results reported between different groups within the literature have been inconsistent and our results were

also variable, suggesting that the mammosphere assay is not a robust assay and not suitable for this purpose. Other methods are required such as evaluation of a wider panel of stem cell markers, investigation of cell surface markers and use of in vivo methods such as transplantation paradigms or lineage tracing experiments. Regulation of stem cell signalling by Kindlin-1 could be important for tumour initiation and outgrowth of micrometastases. We found that Kindlin-1 regulates the protein levels of the cytokine IL-6, a protein that has been linked to NF-κB signalling. This suggests a relevant role for Kindlin-1 in potential stem cell signalling. Preliminary analysis suggested that Kindlin-1 may be important for regulation of a transcriptional activator of the canonical NF-κB pathway in the nucleus, p65. It would be useful to investigate this pathway in further detail and evaluate if loss of Kindlin-1 impacts other NF-κB gene targets.

6.2 A role in the anti-tumour immune response

We found evidence that suggests there may be a complex interplay with Kindlin-1 and the immune system. One of the most significant questions in the cancer field is: how do cancer cells evade the immune system? Our data points towards a role in regulation of T cell populations by Kindlin-1 that enables tumour cells to avoid destruction by the immune system. Loss of Kindlin-1 in an immunocompetent mouse model resulted in complete clearance of the tumour, whereas loss of Kindlin-1 in an immunocompromised mouse model that has a reduced amount of T cells (229), did not result in complete clearance of the tumour by the immune system. We show that mechanistically, loss of Kindlin-1 post-transcriptionally upregulates production of the cytokine IL-6, which reduces the population of Tregs and increases the population of Th17 cells. High numbers of Tregs in cancer tissues has been connected with a pro-tumour effect, disease progression and a worse prognosis in breast cancer (371). We propose that loss of Kindlin-1 leads to upregulation of IL-6 and this drives differentiation of naïve CD4+ T cells from Tregs into Th17 cells, which has a protective, anti-tumour function. Conversely, presence of Kindlin-1 results in downregulation of

IL-6 and leads to differentiation of naive CD4+ T cells into Tregs, which suppresses the anti-tumour immune response resulting in tumour growth. The regulation of T cell populations by Kindlin-1 could be relevant for the outgrowth of micrometastases as well as primary tumour growth demonstrated here. Metastasis-initiating cells need to develop mechanisms to evade the immune system in the target organ and the immune-related mechanisms operating at this level are largely unknown. Therefore uncovering the molecular details of Kindlin-1 and the regulation of T cells could help dissect the complex interactions between the immune population and disseminated cancer cells.

We used a proteomics approach to identify novel binding partners of Kidlin-1 to address our third aim and followed up hits that possibly linked Kinlin-1 to regulation of IL-6. This included a subunit of cytoplasmic dynein, however, the results of these experiments were inconclusive. In the context of the immune system, a potential candidate that should be followed up in the future is ALDH18A1. ALDH18A1 is a member of the aldehyde dehydrogenase family and although we did not validate binding of Kindlin-1 to ALDH18A1, this could be relevant in our model of how Kindlin-1 interacts with the immune system. Aldehyde dehydrogenase (ALDH) and its production of retinoic acid (RA) by different immune cells has been implicated in immune tolerance through the induction of Tregs from naïve CD4+ T cells (372,373). RA has also been shown to inhibit differentiation of naive $CD4^+$ T cells into pro-inflammatory Th17 cells (372,373). It is thought that ALDH inhibition may increase the ratio of effector T cells to Tregs and enhance T-cell mediated tumour elimination (374). Overexpression of ALDH has been associated with poor prognosis in various tumour types and its expression has also been found in CSCs and linked to metastasis, (375). Several ALDH inhibitors have been developed with some promise of treating cancer and therefore, if ALDH18A1 is shown to interact with Kindlin-1 it may therefore present a way of targeting Kindlin-1 induced tumorigenesis (376).

6.3 A nuclear role that is important for primary tumour growth

To our knowledge, this is the first study to report Kindlin-1 in the nucleus of cancer cell lines. As Kindlin-2 and other focal adhesion proteins have established nuclear roles in tumorigenesis as scaffolding proteins that form complexes with transcription factors, we hypothesised that Kindlin-1 may also play a similar role in breast cancer cells to regulate factors associated with metastasis. In order to address the final aim of this study, we generated a Kindlin-1 NLS mutant and identified that mutation of certain amino acids in our predicted NLS sequence, sequesters Kindlin-1 in the cytoplasm. This confirms that like Kindlin-2, Kindlin-1 has a functional NLS sequence. Loss of Kindlin-1 localisation to the nucleus significantly reduced primary tumour growth, however, the mechanism that nuclear Kindlin-1 regulates tumour growth has not been established. Immunophenotyping of the tumours from *in vivo* experiments would provide information on the status of the immune system and establish if T cell ratios are impacted with loss of nuclear Kindlin-1. This would be important to establish if Kindlin-1 is transcriptionally controlling factors, which regulate the T cells.

We performed preliminary analysis that suggests that Kindlin-1 may be important for p65 regulation in the nucleus. We found increased levels of nuclear p65 with presence of nuclear Kindlin-1 when cells were stimulated with TNFα. IL-6 is regulated by the NF-κB pathway and nuclear NF-κB p65 induces IL-6 gene expression (369). With increased nuclear p65, we would expect increased expression of IL-6, however, we found that Kindlin-1 inhibits IL-6 expression. Our data suggests that Kindlin-1 does not regulate IL-6 expression via the NF-κB pathway and we propose that Kindlin-1 post-transcriptionally regulates IL-6 by affecting the stability or degradation of the protein. Our finding that Kindlin-1 may the NF-κB pathway is novel and additional NF-κB gene targets need to be evaluated to determine if Kindlin-1 regulates the NF-κB pathway as this could have implications in both the immune response and stem cell signalling.

6.4 Wider implications

The results presented in this thesis have focused on the role of Kindlin-1 in primary breast cancer growth. As described above, we believe that the roles that we have uncovered for Kindlin-1 in primary tumour growth, may be important for promoting pulmonary breast cancer metastasis and may also have wider implications in other cancer types and inflammatory disease.

6.4.1 Implications in different tumour types

As well as breast cancer, increased expression of Kindlin-1 has been found to promote colon (116) and pancreatic (184) cancers. Kindlin-1 may also have similar roles in these cancer types to the roles we have identified here. Intriguingly, Kindlin-1 appears to have a tumour suppressor function in lung (186), oesophageal (182) and skin cancer (187). Patients with KS are predisposed to SCC and previous research by the Brunton group found that re-expression of WT Kindlin-1 in a mouse SCC model inhibited tumour growth (210). Our data showed that Kindlin-1 localises to the nucleus of the SCC WT Kindlin-1 re-expressed cells (Chapter 3). It is therefore possible that Kindlin-1 could have opposing nuclear roles in cancers where its expression inhibits tumour growth and regulates genes that inhibit rather than promote cancer.

6.4.2 Implications in inflammatory disease

Patients with Kindler syndrome also suffer from an ulcerative colitis-like condition (149,377). Ulcerative colitis (UC) is an inflammatory bowel disease that is a consequence of perturbed immune homeostasis. UC is stimulated by a misbalance in cytokine production and various types of immune cells have a crucial role in pathogenesis by regulation, suppression, and maintenance of inflammation. Importantly, Th17 cells have been shown to be important regulators of inflammatory bowel disease as antibody-mediated blockade of IL-6 (an important driver of differentiation of CD4+ T cells to Th17 cells, as we have previously described in

chapter 3), reduced experimental colitis (378,379). Additionally, a number of polymorphisms in genes linked to Th17 cells have been connected with inflammatory bowel disease (380). The potential role that we have uncovered for Kindlin-1 in relation to IL-6 and T cell regulation could therefore be important for the UC-like symptoms of KS patients. IL-6 has also been linked to progressive fibrosis of the dermis in KS (180) and our findings could also be relevant here.

6.5 Summary and conclusion

In conclusion, the work presented here suggests that Kindlin-1 has an integrin-dependent role in tumour initiation but once the tumour has been established, Kindlin-1 has roles independent of integrin activation that are important for tumour growth. We show that the immune system impacts on its ability to regulate tumour growth and our evidence suggests a potential role for Kindlin-1 in the regulation of T cell subset changes. Our data suggest that one mechanism by which Kindlin-1 may control the ratios of T cells is through the regulation of IL-6 protein levels. We propose that reduction of IL-6 drives differentiation of naïve CD4+ T cells into T regulatory cells, which suppresses the anti-tumour immune response and promotes tumorigenesis. We also found that nuclear Kindlin-1 is important for tumour growth and similar to Kindlin-2, Kindlin-1 also has an NLS that tags it for nuclear import. Given that Kindlin-1 is a well-established focal adhesion protein whose most researched role is in relation to integrin activation, it is interesting to find evidence that suggests Kindlin-1 has important functions in the nucleus. Questions stemming from this work include whether Kindlins' nuclear function is also important for the regulation of the immune system and if Kindlin-1 regulates pathways linked to tumourigenesis through gene regulatory mechanisms.

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